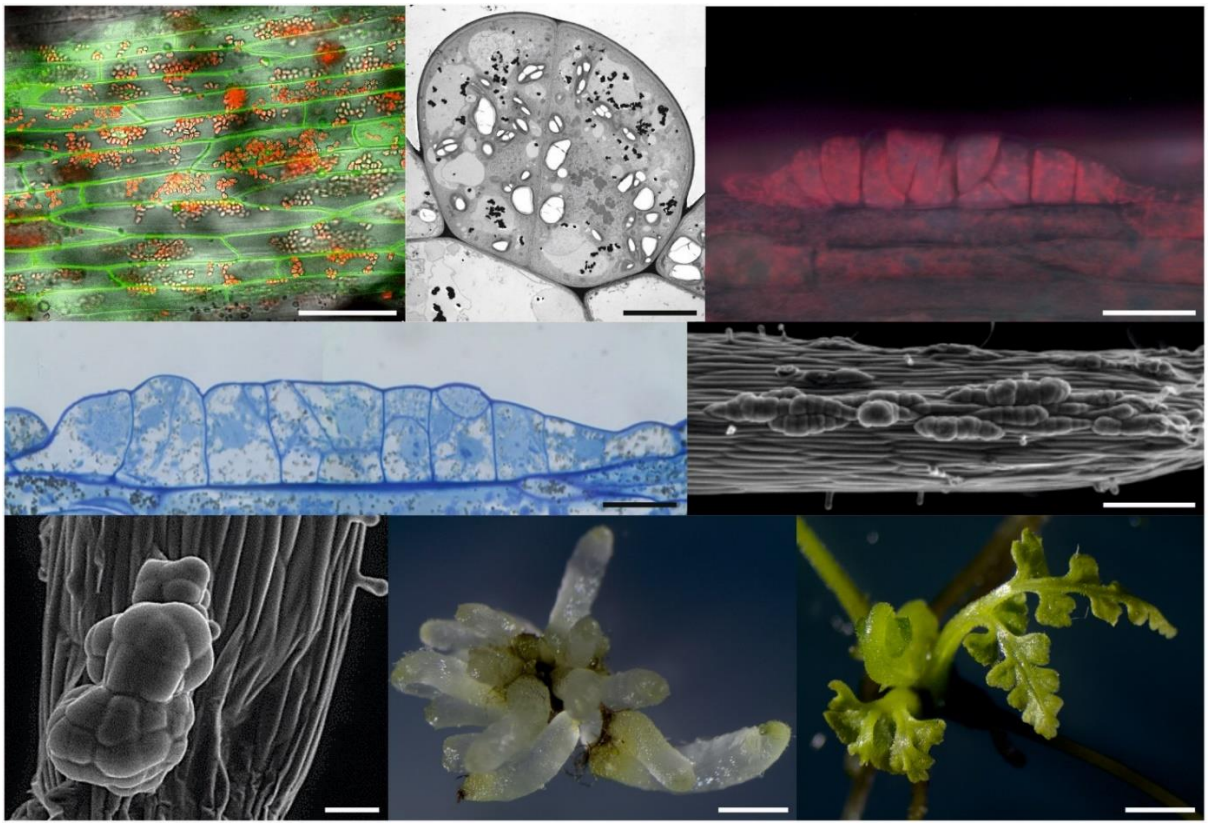
Rozprawa doktorska

Polska Akademia Nauk Ogród Botaniczny – Centrum Zachowania Różnorodności Biologicznej w Powsinie

Cytomorfologiczne i fizjologiczne przemiany leżące u podstaw nabywania embriogenicznej kompetencji przez eksplantaty paproci drzewiastej *Cyathea delgadii* Sternb.



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Promotor pomocniczy: dr Justyna Wróbel-Marek

Rozprawa przedłożona Radzie Instytutu Biologii, Biotechnologii i Ochrony Środowiska Uniwersytetu Śląskiego jako podstawa uzyskania stopnia naukowego doktora

Przedstawione w pracy badania zostały sfinansowane ze środków Narodowego Centrum Nauki w ramach grantów PRELUDIUM 14 nr 2017/27/N/NZ3/00434 i OPUS 2 nr 2011/03/B/NZ9/02472 oraz środków na działalność statutową PAN OB - CZRB w Powsinie

Warszawa, 2020

Opis zdjęć ze strony tytułowej

Od komórki epidermy do kompletnej rośliny – przebieg somatycznej embriogenezy u paproci drzewiastej *Cyathea delgadii* Sternb. zobrazowany różnymi technikami mikroskopowymi.

Górny rząd od lewej:

1. Komórki epidermy inicjalnego eksplantatu ogonka liściowego. Widoczna czerwona autofluorescencja chlorofilu i zielona autofluorescencja ściany komórkowej wzbudzona pod wpływem światła o długości fali 340–380 nm; preparat przyżyciowy; mikroskop laserowy konfokalny (Leica TCS SP5 II); skala 50 µm.
2. Przekrój poprzeczny przez dwie komórki liniowego somatycznego zarodka; 12 dzień kultury, preparat trwały, skrawek grubości 90 nm; transmisyjny mikroskop elektronowy (FEI268D „Morgagni"); skala 25 µm; (Grzyb i in. 2020).
3. Wielokomórkowy somatyczny zarodek. Widoczna czerwona autofluorescencja chlorofilu w świetle UV (filtr BV: 400–440 nm); 12 dzień kultury, preparat przyżyciowy; mikroskop świetlny (Vanox, Olympus); skala 50 µm; (Tomiczak i in. 2018).

Środkowy rząd od lewej:

4. Przekrój podłużny przez somatyczny zarodek w stadium liniowym wybarwiony błękitem toluidyny; 14 dzień kultury, preparat trwały, skrawek grubości 2 µm; mikroskop świetlny (Vanox, Olympus); skala 25 µm; (Grzyb i in. 2019).
5. Liczne somatyczne zarodki w stadium liniowym; 14 dzień kultury, preparat przyżyciowy; środowiskowy skaningowy mikroskop elektronowy (FEI QUANTA 200); skala 250 µm; (Grzyb i in. 2019).

Dolny rząd od lewej:

6. Somatyczny zarodek z widoczną segmentową budową; 21 dzień kultury, preparat przyżyciowy; środowiskowy skaningowy mikroskop elektronowy (FEI QUANTA 200); skala 100 µm; (Grzyb i in. 2020).
7. Młodociane sporofity uzyskane na drodze somatycznej embriogenezy z pojedynczych komórek epidermy eksplantatu ogonka liściowego po 2 miesiącach kultury prowadzonej w ciemności; mikroskop świetlny stereoskopowy (SZH, Olympus); skala 1 mm.
8. Sporofit z rozwiniętymi liśćmi i licznymi korzeniami uzyskany poprzez utrzymywanie roślin widocznych na poprzednim zdjęciu w warunkach fotoperiodu (16/8 h światło/ciemność) przez 3 miesiące; mikroskop świetlny stereoskopowy (SZH, Olympus) ; skala 1 mm.

Podziękowania

Pragnę złożyć najszczersze podziękowania wszystkim, którzy przyczynili się do powstania tej pracy.

Promotorowi rozprawy - ***Pani Profesor Annie Mikule*** *dziękuję za możliwość pracy z niezwykle pasjonującym obiektem badawczym. Nieoceniona pomoc i opieka Pani Profesor, okazywane na każdym kroku naukowej drogi, pozwoliły mi odkrywać piękno embriogenezy in vitro paproci.*

Promotorowi pomocniczemu - ***Pani Doktor Justynie Wróbel-Marek*** *dziękuję za wprowadzenie do fascynujących badań nad symplastową komunikacją oraz za czas, energię i wytrwałość poświęcone wspólnej pracy przy mikroskopie konfokalnym.*

Współautorom publikacji wchodzących w skład rozprawy doktorskiej *dziękuję za możliwość podejmowania wyzwań związanych z nowymi technikami badawczymi.*

Panu Profesorowi Janowi Rybczyńskiemu *dziękuję za merytoryczne wsparcie w rozwiązywaniu problemów badawczych oraz wnikliwe i cenne uwagi w trakcie pracy naukowej.*

Panu Dyrektorowi PAN OB – CZRB w Powsinie - dr Pawłowi Kojsowi *dziękuję za umożliwienie pracy i prowadzenia badań w Zespole Biotechnologii Konserwatorskiej.*

Współpracownikom z Ogrodu Botanicznego - ***Karolinie, Lucynie i Wojtkowi*** *dziękuję za wieczny optymizm, dyskusje na różnorodne tematy oraz wsparcie, które umożliwiły pokonywanie trudności stojących na drodze młodego naukowca.*

Szczególne podziękowania składam mojemu Rodzeństwu będącemu źródłem nieustającej inspiracji. Dziękuję za motywację oraz nigdy niegasnącą wiarę we mnie.

Spis treści

1. Wykaz dorobku naukowego

1.1. Oryginalne prace badawcze będące przedmiotem rozprawy doktorskiej

Publikacja nr 1 **Grzyb M**, Kalandyk A, Waligórski P, Mikuła A (**2017**) The content of endogenous hormones and sugars in the process of early somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb. Plant Cell Tissue and Organ Culture 129:387-397. Doi:10.1007/s11240-017-1185-8

IF_{2017} – 2,390; $MNiSW_{2017}$ – 30 pkt

Publikacja nr 2 **Grzyb M**, Kalandyk A, Mikuła A (**2018**) Effect of TIBA, fluridone and salicylic acid on somatic embryogenesis and endogenous hormone and sugar contents in *Cyathea delgadii* Sternb. Acta Physiologiae Plantarum 40:1. Doi:10.1007/s11738-017-2577-4

IF_{2018} – 1,364; $MNiSW_{2018}$ – 25 pkt

Publikacja nr 3 **Grzyb M**, Wróbel-Marek J, Kurczyńska E, Sobczak M, Mikuła A (**2020**) Symplasmic isolation contributes to somatic embryo induction and development in the tree fern *Cyathea delgadii* Sternb. Plant and Cell Physiology. Doi:10.1093/pcp/pcaa058

IF_{2020} – 3,929; $MNiSW_{2020}$ – 140 pkt

Publikacja nr 4 **Grzyb M**, Mikuła A (**2019**) Explant type and stress treatment determine the uni- and multicellular origin of somatic embryos in the tree fern *Cyathea delgadii* Sternb. Plant Cell, Tissue and Organ Culture 136:221-230. Doi:10.1007/s11240-018-1507-5

IF_{2019} – 2,200; $MNiSW_{2019}$ – 100 pkt

Sumaryczny IF prac stanowiących dysertację – 9,883

Sumaryczna punktacja MNiSW – 295 pkt

1.2. Pozostałe oryginalne prace

Artykuły naukowe:

Domżalska L, Kędracka-Krok S, Jankowska U, **Grzyb M**, Sobczak M, Rybczyński JJ, Mikuła A (**2017**) Proteomic analysis of stipe explants reveals differentially expressed proteins involved in early direct somatic embryogenesis of the tree fern *Cyathea delgadii* Sternb. Plant Science 258:61-76. Doi:10.1016/j.plantsci.2017.01.017

IF_{2017} – 3,712; $MNiSW_{2017}$ – 35 pkt

Mikuła A, Pożoga M, **Grzyb M**, Rybczyński JJ (**2015**) An unique system of somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb. - the importance of explant type, and physical and chemical factors. Plant Cell Tissue and Organ Culture 123:467-478. Doi:10.1007/s11240-015-0850-z

IF_{2015} – 2,390; $MNiSW_{2015}$ – 30 pkt

Rozdziały w monografiach:

Tomiczak K, **Grzyb M**, Rybczyński JJ, Mikuła A (**2018**) Somatic embryogenesis and somatic embryo cryopreservation of the tree-fern *Cyathea delgadii* Sternb. In: Jain S., Gupta P. (eds) Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants. Forestry Sciences, vol 85. Springer, Cham, pp 291-303. Doi:10.1007/978-3-319-79087-9_23

$MNiSW_{2018}$ – 8

Rybczyński JJ, Tomiczak K, **Grzyb M**, Mikuła A (**2018**) Morphogenic Events in Ferns: Single and Multicellular Explants In Vitro. In: Fernández H. (eds) Current Advances in Fern Research. Springer, Cham, pp 99-120. Doi:10.1007/978-3-319-75103-0_5

$MNiSW_{2018}$ – 8

Mikuła A, **Grzyb M**, Tomiczak K, Rybczyński JJ (**2018**) Experimental and Practical Application of Fern Somatic Embryogenesis. In: Fernández H. (eds) Current Advances in Fern Research. Springer, Cham, pp 121-137. Doi:10.1007/978-3-319-75103-0_6

$MNiSW_{2018}$ – 8

Sumaryczny IF pozostałego dorobku naukowego – 6,102

Sumaryczna punktacja MNiSW – 89 pkt

1.3. Prezentacje na konferencjach naukowych

a) krajowych

1. XI Konferencja „Kultury in vitro w biotechnologii i fizjologii roślin”; 4-6 grudnia 2019, Kraków:
   - **Grzyb M**, Wróbel-Marek J, Kurczyńska E, Mikuła A. *Cyathea delgadii* jako model w badaniach nad rolą komunikacji symplasmowej w trakcie somatycznej embriogenezy (**wykład**)
   - Mikuła A, **Grzyb M**, Tomaszewicz W, Rybczyński JJ. Wgląd w somatyczną embriogenezę roślin poprzez system eksperymentalny *Cyathea delgadii* (wykład na zaproszenie)
   - Tomaszewicz W, Dziurka M, **Grzyb M**, Mikuła A. Drogi różnicowania somatycznych zarodków paproci drzewiastej *Cyathea delgadii*: strukturalno-fizjologiczne podstawy (wykład)
2. Botanika bez granic – 58. Zjazd Polskiego Towarzystwa Botanicznego; 1–7 lipca 2019, Kraków:
   - **Grzyb M**, Wróbel-Marek J, Kurczyńska E, Mikuła A. Zmiany w komunikacji symplastowej w trakcie somatycznej embriogenezy paproci drzewiastej *Cyathea delgadii* (**wykład plenarny**)
   - Tomaszewicz W, **Grzyb M**, Dziurka M, Biesaga-Kościelniak J, Rybczyński JJ, Mikuła A. Uwarunkowania leżące u podstaw odmiennych dróg różnicowania somatycznych zarodków *Cyathea delgadii* (plakat)
3. XV Ogólnopolska Konferencja Kultur In Vitro i Biotechnologii Roślin; 17 - 20 września 2018, Rogów:
   - **Grzyb M**, Rybczyński JJ, Mikuła A. Single- versus multiple-cell origin of somatic embryos in the tree fern *Cyathea delgadii* (**wykład**)
   - Mikuła A, Tomiczak K, **Grzyb M**, Tomaszewicz W, Rybczyński JJ. Biotechnology in plant diversity conservation (wykład)
   - Tomaszewicz W, **Grzyb M**, Rybczyński JJ, Mikuła A. Effect of sucrose treatment on somatic embryogenesis in *Cyathea delgadii* Sternb (plakat)
4. VI Ogólnopolska Konferencja Naukowa Sekcji Pteridologicznej PTB pt. Polskie badania pteridologiczne w 100-lecie wydania I tomu Flory Polski – przeszłość i przyszłość; 7-8 września 2017, Poznań:
   - **Grzyb M**, Tomiczak K, Rybczyński JJ, Mikuła A. Somatyczna embriogeneza jako alternatywna metoda pozyskiwania paproci (**wykład**)
   - Mikuła A, **Grzyb M**, Tomiczak K, Rybczyński JJ. Stan wiedzy w zakresie wykorzystania krioprezerwacji w ochronie paproci (wykład plenarny)
   - Rybczyński JJ, Tomiczak K, **Grzyb M**, Mikuła A. Procesy morfogenetyczne paproci w warunkach in vitro (wykład)
5. Konferencja naukowa pt. „Anatomia i histogeneza roślin: wczoraj, dziś i jutro.”: 15-17 maja 2017, Rogów:
   - **Grzyb M**, Wróbel-Marek J, Milewska-Hendel A, Kurczyńska E, Rybczyński JJ, Mikuła A. Od komórki epidermy do zarodka somatycznego –

cytomorfologiczne zmiany zachodzące w eksplantatach ogonków liściowych paproci drzewiastej *Cyathea delgadii* (**wykład**)

6. X Ogólnopolska konferencja pt. „Kultury in vitro w fizjologii roślin"; 7-9 grudnia 2016:
   - **Grzyb M**, Mikuła A, Rybczyński JJ, Kalandyk A, Waligórski P. Rola endogennych hormonów i cukrów w nabywaniu embriogenicznych kompetencji u paproci drzewiastej *Cyathea delgadii* (**komunikat ustny**)
7. 57 Zjazd Polskiego Towarzystwa Botanicznego; 27 czerwca – 6 lipca 2016, Lublin:
   - Mikuła A, **Grzyb M**, Kalandyk A, Waligórski P, Rybczyński JJ. Hormonalna równowaga jest podstawą indukcji embriogenicznej kompetencji u paproci drzewiastej *Cyathea delgadii* Sternb. (wykład)
8. XIV Ogólnopolska Konferencja Kultur In Vitro i Biotechnologii Roślin, 14-17 września 2015, Poznań:
   - **Grzyb M**, Tomiczak K, Rybczyński JJ, Mikuła A. Wpływ różnych stężeń sacharozy, pH i inhibitorów hormonów na efektywność somatycznej embriogenezy u dwóch gatunków paproci: *Cyathea delgadii* i *Asplenium cuneifolium* (**komunikat ustny**)
   - Mikuła A, **Grzyb M**, Pożoga M, Tomiczak K, Domżalska L, Rybczyński JJ. Somatyczna embriogeneza paproci drzewiastej *Cyathea delgadii* Sternb.: osiągnięcia i perspektywy (wykład)
   - Wróbel-Marek J, **Grzyb M**, Kurczyńska E, Rybczyński JJ, Mikuła A. Wybrane aspekty symplastowej komunikacji w czasie somatycznej embriogenezy paproci drzewiastej *Cyathea delgadii* (plakat)

b) międzynarodowych

9. Integrative Plant Biology Conference; 7-9 listopada 2018, Poznań:
   - **Grzyb M**, Tomaszewicz W, Rybczyński JJ, Mikuła A. The progress in research on fern somatic embryogenesis (**plakat**)
10. 11th International Conference "Plant Functioning Under Environmental Stress"; 12-15 września 2018, Kraków:
   - **Grzyb M**, Tomaszewicz W, Rybczyński JJ, Mikuła A. The endo- and exogenous regulation of somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb. - the influence of sucrose (**plakat**)
11. 8th Conference of the Polish Society of Experimental Plant Biology Communication in plants: from cell to environment; 12-15 września 2017, Białystok:
   - **Grzyb M**, Wróbel-Marek J, Kurczyńska E, Rybczyński JJ, Mikuła A. Symplasmic communication during development of *Cyathea delgadii* somatic embryos (wykład)
12. Plant Biology Europe EPSO/FESPB 2016 Congress; 26-30 czerwca 2016, Prague, Czech Republic:
   - **Grzyb M**, Kalandyk A, Waligórski P, Rybczyński JJ, Mikuła A. Changes in endogenous hormone and sugar contents during somatic embryogenesis induction in the tree fern *Cyathea delgadii* (Sternb.) (plakat)

13. 6th International Symposium on Production and Establishment of Micropropagated Plants; 19-24 kwietnia 2015, Sanremo:
    - **Grzyb M**, Pożoga M, Rybczyński JJ, Mikuła A. The first insight into endogenous hormonal stimulation of somatic embryogenesis in tree-fern *Cyathea delgadii* (Sternb.) (**plakat**)
    - Tomiczak K, **Grzyb M**, Pożoga M, Rybczyński JJ, Mikuła A. The influence of various sucrose concentrations and pH on efficiency of somatic embryogenesis in two fern species: *Cyathea delgadii* and *Asplenium cuneifolium* (plakat)
    - Mikuła A, Pożoga M, **Grzyb M**, Rybczyński JJ. The influence of explant type, lighting conditions and salt concentrations of MS medium on efficiency of somatic embryogenesis in *Cyathea delgadii* (Sternb.) (plakat)
14. Post-transcriptional Gene Expression Regulation in Plants 30 czerwca – 2 lipca 2014, Poznań:
    - Szczygiel-Sommer A, **Grzyb M**, Szyrajew K, Nowak K, Gaj MD. miR172 is involved in control of somatic embryogenesis induced in vitro in Arabidopsis (plakat)

1.4. Realizowane projekty badawcze

1. Projekt badawczy (2-letni) pt. Rola komunikacji międzykomórkowej w indukcji somatycznej embriogenezy i formowaniu zarodka somatycznego w oparciu o modelowy system *Cyathea delgadii* Sternb. (The role of intercellular communication in somatic embryogenesis induction and somatic embryo formation based on a model of *Cyathea delgadii* Sternb.) 2017/27/N/NZ3/00434; Realizacja w latach: 2018-2020 (140 000 zł); **kierownik projektu**
2. Projekt badawczy (3-letni) nr UMO-2011/03/B/NZ9/02472 pt. „Somatyczna embriogeneza paproci – nowe zjawisko w biologii eksperymentalnej roślin. Analiza cytomorfologiczno-proteomiczna i krioprezerwacja", **wykonawca** w latach 2014 - 2015

1.5. Udział w szkoleniach i stażach

1. Szkolenie „Wprowadzenie do obróbki i analizy danych NGS" przeprowadzone przez Ideas4biology Sp. Z o.o.; 09.03.2018, Poznań.
2. Staż naukowy „Zbadanie roli komunikacji symplastowej w formowaniu zarodków z komórek somatycznych epidermy u *Cyathea delgadii*". Badania w ramach stażu obejmowały wykorzystanie niskocząsteczkowych fluorochromów transportu symplastowego i technikę mikroskopii konfokalnej. Katedra Biologii Komórki, Wydział Biologii i Ochrony Środowiska, Uniwersytet Śląski, 17.10-12.11 2016, Katowice.
3. Staż naukowy „Zobrazowanie kolejnych stadiów rozwojowych zarodka somatycznego *Cyathea delgadii* z wykorzystaniem transmisyjnej mikroskopii elektronowej (TEM)".

Katedra Biologii Komórki, Wydział Biologii i Ochrony Środowiska, Uniwersytet Śląski, 04.04.2016 do 27.05.2016., Katowice.

4. Szkolenie „Wykorzystanie mikroskopii konfokalnej i barwników fluorescencyjnych w opisie procesu indukcji i ekspresji somatycznej embriogenezy". Katedra Biologii Komórki, Wydział Biologii i Ochrony Środowiska, Uniwersytet Śląski, 23 - 31.03.2015, Katowice.

1.6. Nagrody, stypendia i wyróżnienia

- Nagroda Wydziału II Nauk Biologicznych i Rolniczych PAN z dnia 21.11.2019r. dla Zespołu Naukowego z PAN Ogrodu Botanicznego-CZRB w Powsinie prof. dr hab. Anna Mikuła, mgr Małgorzata Grzyb, dr Lucyna Domżalska, prof. dr hab. Jan Rybczyński za cykl prac pt. „Odkrycie i rozwinięcie systemu modelowego somatycznej embriogenezy u roślin z kladu Monilophyta w warunkach in vitro"
- Nagroda Polskiego Towarzystwa Botanicznego dla młodych pracowników nauki przyznana za serię opublikowanych w latach 2017-2019 artykułów naukowych opisujących wczesne etapy indukcji somatycznej embriogenezy u paproci drzewiastej *Cyathea delgadii* Sternb. na poziomie cytomorfologicznych i fizjologicznych zmian zachodzących w eksplantatach inicjalnych; 58 Zjazd Polskiego Towarzystwa Botanicznego, Kraków, 2 lipca 2019 r.
- Nagroda Naukowa Dyrektora PAN Ogrodu Botanicznego – CZRB w Powsinie za wyróżniający się dorobek publikacyjny w 2018 roku
- Nagroda w konkursie na najlepsze wystąpienie ustne pod tytułem: "Od komórki epidermy do zarodka somatycznego - cytomorfologiczne zmiany zachodzące w eksplantatach ogonków liściowych paproci *Cyathea delgadii*" na Ogólnopolskiej Konferencji Anatomii i Histogenezy roślin - wczoraj, dziś i jutro. Rogów 16-17 maja 2017; Rogów
- Wyróżnienie w konkursie na najlepszą prezentacje plakatową plakatu pt. „Wybrane aspekty symplastowej komunikacji w czasie somatycznej embriogenezy paproci drzewiastej *Cyathea delgadii*" autorstwa: Wróbel-Marek J, Grzyb M, Kurczyńska E, Rybczyński JJ, Mikuła A, w ramach XIV Ogólnopolskiej konferencji kultur in vitro i biotechnologii roślin. „Strukturalne, fizjologiczne i molekularne podstawy różnicowania roślin". Poznań, 14-17 września 2015

2. Streszczenie

Somatyczna embriogeneza (SE), będąca procesem formowania zarodków z komórek wegetatywnych, jest przejawem niezwykłej plastyczności organizmów roślinnych, która fascynuje badaczy od ponad 60 lat. Pomimo ogromnego postępu jaki w tym czasie osiągnięto, kwestia jak pojedyncza komórka somatyczna zmienia swój program rozwojowy by stać się kompletną rośliną do tej pory nie została dostatecznie wyjaśniona. Opracowany w 2015 r. system eksperymentalny SE u paproci *Cyathea delgadii*, dzięki jednokomórkowemu pochodzeniu zarodków indukowanych na pożywkach bez użycia roślinnych regulatorów wzrostu, otworzył drogę do prowadzenia badań nad etapem indukcji i wczesnej ekspresji tego procesu. Celem dysertacji było poznanie i opisanie uwarunkowań związanych z nabywaniem embriogenicznego potencjału przez eksplantaty *C. delgadii* na poziomie struktury i ultrastruktury komórek, fizjologicznych zależności oraz symplastowej komunikacji. Obiektem prowadzonych eksperymentów była jedyna jak dotąd paproć, dla której opisano zdolność rozmnażania na drodze SE. Analizom poddano eksplantaty ogonków liściowych i międzywęźli pochodzące z etiolowanych sporofitów *C. delgadii* oraz ogonków pobieranych z nieetiolowanych roślin.

Wykorzystując wysokosprawną chromatografię cieczową wykazano, że warunki świetlne, w których rozwijają się donorowe rośliny wpływają znacząco na poziom i równowagę endogennych hormonów i cukrów. Udowodniono, że kwas abscysynowy (ABA) jest głównym hormonem hamującym SE, a nagły wzrost stężenia endogennej sacharozy jest sygnałem indukującym zmianę programu rozwojowego somatycznych komórek. Stosując inhibitory transportu i biosyntezy hormonów wykazano, że substancje te w stężeniach hamujących SE istotnie zmieniają poziom endogennego kwasu indolilo-3-octowego (IAA) i/lub ABA i/lub CK. Ich zastosowanie skutkuje także zmianą w koncentracji endogennych cukrów, zwłaszcza kluczowej dla indukcji tego procesu sacharozy. Eksperymenty przeprowadzone z wykorzystaniem niskocząsteczkowych fluorochromów transportu symplastowego wykazały ograniczenie w łączności symplastu w eksplantacie tuż przed wystąpieniem podziałów dających początek somatycznym zarodkom. Zmianom w ciągłości cytoplazmy towarzyszyła jej przebudowa w komórkach epidermy i kory obserwowana z użyciem różnych technik mikroskopowych. Rozwój somatycznego zarodka był również związany z ograniczeniem przemieszczania fluorochromów pomiędzy jego komórkami a eksplantatem, jak również w obrębie ciała zarodka. Analizując szereg czynników kultury mogących wpływać na przebieg i efektywność SE, stwierdzono, że w zależności od typu eksplantatu użytego do inicjacji kultury, somatyczne zarodki *C. delgadii* powstają na drodze jedno- (na eksplantacie ogonka) lub wielokomórkowej (na eksplantacie międzywęźla). Poprzez krótkotrwałe traktowanie międzywęźli roztworami sacharozy można wielokrotnie podnieść efektywność SE, co jest związane ze zmianą drogi różnicowania zarodków z wielo- w jednokomórkową.

Wieloaspektowe badania dostarczyły nowej, kompleksowej wiedzy w zakresie przemian towarzyszących przejściu komórki somatycznej eksplantatu w stan embriogeniczny oraz związanych z wczesnym etapem różnicowania ciała zarodka paproci.

3. Streszczenie w języku angielskim

Somatic embryogenesis (SE) is the formation of embryos from vegetative cells. This process, as an example of the extraordinary plasticity of plant organisms, has fascinated researchers for over 60 years. Despite the enormous progress made during this time, the question of how a single somatic cell changes its developmental program to become a complete plant has not been sufficiently clarified so far. Experimental system developed in 2015 in tree fern *Cyathea delgadii* is characterized by single-cell origin of somatic embryos induced on media without plant growth regulators. These particular qualities has opened the way for research on the induction and early expression of SE. The aim of the dissertation was to describe determinants related to the acquisition of embryogenic potential by *C. delgadii* explants at the level of cell structure and ultrastructure, physiological relationships and symplasmic communication. The experiments were conducted on *C. delgadii*, the one fern in which SE was discovered so far. Stipe and internode explants excised from etiolated sporophyte and stipe explants taken from non-etiolated plants, were analyzed.

High-performance liquid chromatography analysis shown that the light conditions in which donor plants develop significantly affect the level and balance of endogenous hormones and sugars. It also proved that abscisic acid (ABA) is the main hormone inhibiting SE and sudden increase in the concentration of endogenous sucrose is a signal inducing developmental change of somatic cells. Hormone transport and biosynthesis inhibitors used in SE-inhibitory concentrations, significantly changed the level of endogenous IAA and/or ABA and/or CK. Their application also caused change in the concentration of endogenous sugars, especially critical for SE induction, sucrose. Experiments with low molecular weight fluorochromes of symplasmic transport have shown the restriction in the cellular communication of stipe explant just before the divisions giving rise to somatic embryos occurred. The changes in the cytoplasm flow were accompanied by remodeling in epidermal and cortical cells structure observed using various microscopic techniques. The development of the somatic embryo was also associated with the restriction of fluorochromes movement between its cells and the explant as well as within the embryo body. By testing a number of factors that may influence the course and efficiency of SE, it was found that depending on the type of explant used for culture initiation, somatic embryos of *C. delgadii* were uni- (on the stipe explants) or multi-cellular (on the interstitial implantation) in origin. Short term treatment of internodes with sucrose solutions increased the SE efficiency by many times. That was related to the change of the embryo differentiation pathway from multi-cellular to uni-cellular.

Studies provided new, comprehensive knowledge on the cellular changes accompanying the transition of somatic cell to the embryonic state and related to the early stage of differentiation of the fern embryo.

4. Wstęp

Somatyczna embriogeneza (SE) jest procesem formowania zarodków z komórek wegetatywnych, które w rozwoju rośliny nie są do tego predysponowane. Powstające tą drogą dwubiegunowe struktury wykształcają własny system waskularny niepołączony z tkanką przewodzącą inicjalnego eksplantatu. Zarodki te, zwane somatycznymi, różnicują wprost z komórek eksplantatu lub poprzez przejściową fazę kalusa (droga bezpośrednia lub pośrednia), a ich rozwój jest inicjowany podziałami pojedynczej komórki bądź wielu sąsiadujących ze sobą komórek (pochodzenie jedno- lub wielokomórkowe) (Maheswaran i Williams 1985). Formowanie somatycznych zarodków jest szczególnym przykładem plastyczności organizmów roślinnych, bazującym na zmianie dotychczas realizowanego programu rozwojowego (Elhiti i in. 2013). Taka reorganizacja w warunkach kultury in vitro zachodzi zazwyczaj pod wpływem egzogennych roślinnych regulatorów wzrostu (ang. plant growth regulators; PGRs), rzadziej jedynie po zastosowaniu czynników stresowych takich jak np. mechaniczne uszkodzenie, traktowanie wysoką lub niską temperaturą, metalami ciężkimi czy stresem osmotycznym (Loyola-Vargas i Ochoa-Alejo 2016; Nic-Can i in. 2016). Podwaliny kulturom tkankowym roślin, w tym SE, dał austriacki botanik Gottlieb Haberlandt (1854-1945), który w 1902 roku sformułował śmiałą hipotezę mówiącą, że każda żywa komórka somatyczna jest zdolna do odtworzenia kompletnego organizmu roślinnego (Haberlandt 1902). Ponad pół wieku później dwóch badaczy - Reinert i Steward, prowadząc niezależne badania, opisało powstawanie zarodków z kultur zawiesin komórkowych *Daucus carota* (Steward i in. 1958; Reinert 1959). W ciągu nieco ponad dwudziestu lat od odkrycia SE, proces ten został udokumentowany u 132 gatunków roślin nago- i okrytozalążkowych, jedno- i dwuliściennych (Ammirato 1983). Ostatnie opracowanie, w którym podsumowano liczbę gatunków zdolnych do regeneracji tą drogą, pochodzi z 1995 r. (Thorpe 1995). Obecnie, ze względu na dobrze poznane warunki kultury i czynniki kontrolujące przebieg SE, a także wysoką efektywność tego procesu, jest on wykorzystywany na dużą skalę w klonalnym rozmnażaniu roślin i znajduje szerokie zastosowanie w ogrodnictwie, leśnictwie, rolnictwie i medycynie. Z naukowego punktu widzenia współczesne dociekania nad SE zmierzają w kierunku poznania i opisania mechanizmów sterujących embriogenezą roślin (Elhiti i in. 2013; Fehér 2015).

Mimo ponad 60-letniego doświadczenia w odkrywaniu SE, wiedza na jej temat pochodzi całkowicie z badań prowadzonych nad Spermatophyta. Poza grupą roślin nasiennych proces ten opisano dotychczas jedynie u dwóch gatunków widłaków tj. *Lycopodiella inundata* (L.) Holub (Atmane i in. 2000) i *Huperzia selago* (L.) (Szypuła i in. 2005). Ich systemy regeneracyjne bazują na pośredniej SE indukowanej dodawanymi do pożywki regulatorami wzrostu. W 2015 r., w naszym laboratorium, opisano po raz pierwszy na świecie SE u paproci drzewiastej *Cyathea delgadii* (klad Monilophyta). To odkrycie wypełniło lukę w poznawaniu tego procesu u różnych filogenetycznie grup roślin telomowych (Mikuła i in. 2015b). Opracowany u *C delgadii* system, dzięki wysokiej efektywności i możliwości otrzymywania somatycznych zarodków bez stosowania PGRs, otworzył drogę do prowadzenia badań w obszarach niedostępnych dotychczas nawet u roślin modelowych. Warto podkreślić, że w ostatnich latach zwrócono uwagę na potencjał roślin zarodnikowych w badaniu podstawowych procesów rozwojowych in vitro. Analizy przeprowadzone

z wykorzystaniem paproci zielnej *Adiantum capillus-veneris* wykazały aktywność genów takich jak *SERK* (*SOMATIC EMBRYOGENESIS RECEPTOR KINASES*), *WUS* (*WUSHEL*), *LEC1* (*LEAFY COTYLEDON 1*), *AGL15* (*AGAMOUS LIKE-15*) i *BBM* (*BABY BOOM*) podczas regeneracji pędów, formowania struktur zwanych (z ang.) green globular bodies i produkcji kalusa (Li i in. 2015; 2017). Działanie kluczowych regulatorów SE w różnych systemach regeneracyjnych paproci skłania do poszukiwania wspólnych dla całego świata roślinnego mechanizmów warunkujących proces różnicowania komórek in vitro.

Wieloletnie badania prowadzone z wykorzystaniem różnych roślin, w tym gatunków modelowych takich jak marchew, lucerna, kukurydza, ryż, bawełna czy rzodkiewnik, doprowadziły do opisania dróg pochodzenia i rozwoju somatycznych zarodków. Umożliwiły również poznanie podstawowych uwarunkowań hormonalnych, genetycznych, epigenetycznych, biochemicznych czy strukturalnych zaangażowanych w proces SE (Fehér 2015; Méndez-Hernández i in. 2019; Kumar i van Staden 2017; Horstman i in. 2017). Mimo ogromnego postępu, współczesna nauka wciąż nie posiada jednak pełnego obrazu przemian komórki somatycznej prowadzących do uzyskania przez nią embriogenicznego charakteru. Modele roślinne, które pozwalają śledzić np. molekularne mechanizmy zachodzące w SE, nie zawsze są optymalne do prowadzenia badań na innych poziomach np. fizjologicznym czy cytomorfologicznym. Dlatego też poszukiwanie i wprowadzanie do badań nowych modeli roślinnych może być niezwykle cenne z punktu widzenia potrzeby pogłębiania wiedzy i budowania kompleksowego obrazu procesu SE, a zwłaszcza jego najwcześniejszej fazy zwanej fazą indukcji (Elhiti i in. 2013).

4.1. Fizjologiczne aspekty indukcji SE

Reakcja na stres i odpowiedź na poziomie hormonalnym inicjowane są jako pierwsze w czasie odróżnicowania komórek w obrębie eksplantatów, umożliwiając im przeprogramowanie i podążanie szlakiem SE (Ikeuchi i in. 2017; Mozgová i in. 2017). Dlatego też, zrozumienie fizjologicznych zależności jest kluczowe w poznaniu podstaw embriogenicznego potencjału i inicjacji somatycznych zarodków (Jimenez 2005; Méndez-Hernández i in. 2019). W większości dotychczas opublikowanych protokołów indukowania SE wykazano kluczową rolę PGRs. Tylko dla niewielkiej liczby roślin możliwe jest inicjowanie tego procesu bez obecności tych substancji w pożywce (Gaj 2004; Karami i Saidi 2010). Systemy SE u wielu gatunków są auksyno-zależne, bazujące na wykorzystaniu syntetycznej auksyny takiej jak kwas 2,4-dichlorofenoksyoctowy (2,4-D; Gaj 2004; Karami i in. 2009). Stąd, endogenne auksyny stanowią grupę hormonów najszerzej analizowaną w kontekście indukowania SE. Obok nich, także cytokininy są niezbędne w fazie wstępnych podziałów komórek w tym procesie (Karami i in. 2009). Etylen i kwas abscysynowy (ABA), uważane za hormony stresu, mogą również być zaangażowane w nabywanie kompetencji do embriogenezy (Karami i Saidi 2010; Zheng i in. 2013; Nowak i in. 2015). W badaniach nad hormonalną regulacją SE znacznie więcej uwagi poświęca się rezultatom dodawania PGRs do pożywki niż możliwej roli fitohormonów obecnych w tkankach. Niewiele też wiadomo o interakcjach egzogennych regulatorów wzrostu z endogennym układem hormonalnym (Ayil-Gutiérrez i in. 2013). Wiedza o zawartości fitohormonów w trakcie indukcji SE najczęściej jest ograniczona do analizy jednej bądź dwóch grup związków (Grieb i in. 1997;

Charrière i in. 1999; Jiménez i Bangerth 2001b; Yang i in. 2012; Ayil-Gutiérrez i in. 2013; Igielski i Kępczyńska 2017; Wójcikowska i in. 2018). Ponadto badania te obejmują głównie porównanie kalusa embriogenicznego z nieembriogenicznym (Jiménez i Bangerth 2000; Pintos i in. 2002) bądź eksplantatów czy genotypów zdolnych lub niezdolnych do SE (Wenck i in. 1988; Centeno i in. 1997; Limanton-Grevet i in. 2000; Jiménez i Bangerth 2001a). Najnowsze doniesienia oparte o analizę bardzo szerokiego spektrum endogennych hormonów u *Picea abies*, ujawniły, że stężenia poszczególnych klas fitohormonów różnią się zasadniczo między fazami SE: proliferacją, dojrzewaniem i kiełkowaniem zarodków (Vondrakova i in. 2018).

Mimo że fizjologiczne aspekty procesu SE są bardzo ważnym i często podejmowanym zagadnieniem, to badaniom tym sporadycznie poddawana jest faza indukcji, w której dochodzi do przeprogramowania komórek somatycznych w embriogeniczne. Ze względu na brak odpowiednich modeli eksperymentalnych badania na tym etapie są niezwykle trudne do przeprowadzenia. Nie sprzyjają temu różne drogi różnicowania zarodków, których powstawaniu towarzyszy formowanie tkanki kalusowej lub strefy komórek przejściowych pociągające za sobą odmienne wzorce odpowiedzi hormonalnej i przemian cukrowych. Z tego względu wiedza o tym etapie SE jest bardzo ograniczona.

4.2. Symplastowa komunikacja w procesie SE

Symplast, będący częścią systemu wymiany informacji między komórkami roślinnymi, jest rozważany jako jeden z ważnych elementów warunkujących uruchamianie procesów morfogenetycznych (Marzec i Kurczynska 2014; Otero i in. 2016). Symplastowa komunikacja może również pełnić kluczową rolę w inicjowaniu SE. Eksperymenty w tym zakresie początkowo skupiały się na zrywaniu plasmodesmowych połączeń inicjowanym roztworami plazmolizującymi, oraz na wpływie takiego traktowania na podniesienie efektywności produkcji somatycznych zarodków (Choi i Soh 1997; Choi i in. 1999). Analizy mikroskopowe plasmodesm i wykazanie zmian w ich liczbie potwierdziły rolę łączności symplastu w SE (Verdeil i in. 2001). Badania prowadzone w ostatnich latach pokazują jednak, że obecność plasmodesm sama w sobie nie świadczy o funkcjonalności symplastu. Ich przepustowość może być regulowana przez wiele czynników takich jak odkładanie β-1,3-glukanu (kalozy; Chen i Kim 2009; Zavaliev i in. 2011) czy aktywność błonowych białek plasmodesmalnych (Burch-Smith i in. 2012; Burch-Smith i Zambryski 2012). Znacznie więcej informacji o wymianie cytoplazmy oraz zawartych w niej cząsteczkach sygnalnych (takich jak hormony, czynniki transkrypcyjne i niektóre RNA) można uzyskać prowadząc analizy oparte na stosowaniu fluorochromów transportu symplastowego. Substancje te, przemieszczając się jedynie plasmodesmami, pozwalają na śledzenie przepływu cytoplazmy pomiędzy komórkami. Są szeroko wykorzystywane w badaniach procesów różnicowania komórek roślinnych takich jak rozwój włośników w korzeniach jęczmienia (Marzec i in. 2014), rozwój zygotycznych zarodków (Kim i Zambryski 2005; Stadler i in. 2005; Wróbel-Marek i in. 2016) oraz w androgenezie (Wróbel i in. 2011). SE z wykorzystaniem fluorochromów była jak dotąd badana zaledwie u jednego gatunku z grupy roślin nasiennych, tj. modelowego *Arabidopsis thaliana.* W badaniach tych wykazano ograniczenie symplastowej komunikacji pomiędzy regionami eksplantatu, z którego różnicują somatyczne zarodki

a obszarami niezaangażowanymi w jego powstawanie (Kulinska-Lukaszek i Kurczynska 2012; Godel-Jedrychowska i in. 2019). Pokazano także czasową izolację somatycznego zarodka w stadium sercowatym od eksplantatu, z którego powstał (Kurczyńska i in. 2007).

Od dawna postuluje się, że izolacja komórki somatycznej, w tym na drodze symplastu, jest warunkiem niezbędnym do przeprogramowania jej dotychczasowego rozwoju i skierowania na ścieżkę SE. Kwestia ta nie została jednak jak dotąd udokumentowana w modelu jednokomórkowego różnicowania zarodków.

4.3. *Cyathea delgadii* jako unikatowy model eksperymentalny

Badania przeprowadzone dotychczas z wykorzystaniem tropikalnego gatunku paproci drzewiastej *C. delgadii* wykazały dwie specyficzne cechy procesu SE, które nadają mu unikatowy eksperymentalny charakter (Mikuła i in. 2015a, b). Należą do nich: 1) indukcja i rozwój zarodków zachodzące na pożywkach bez udziału PGRs i 2) jednokomórkowe, epidermalne pochodzenie somatycznych zarodków.

Paproć *C. delgadii* należy do nielicznej grupy roślin, u których SE można indukować bez stosowania w pożywce PGRs (Raemakers i in. 1995; Gaj 2004; Yan-Lin i Soon-Kwan 2012). Zdolność inicjalnych eksplantatów do formowania somatycznych zarodków zależy u tego gatunku od warunków świetlnych, w jakich rozwijają się donorowe rośliny. Wykazano, że eksplantaty pobierane z roślin rosnących w warunkach fotoperiodu (16/8h) są niezdolne do produkcji somatycznych zarodków, podczas gdy na eksplantatach pochodzących z roślin utrzymywanych w warunkach ciągłej ciemności zarodki powstają z wysoką efektywnością (Mikuła et al. 2015a). Wydajnością SE u *C. delgadii* oraz rozwojem zarodków można sterować za pomocą zawartości mikro- i makroelementów pożywki, stężenia sacharozy oraz warunków świetlnych kultury. Analizy cytomorfologiczne pokazały, że pierwszych 8-10 podziałów zachodzących w obrębie inicjalnej komórki epidermy eksplantatu ogonka liściowego prowadzi do powstania liniowej struktury zarodkowej, w której następnie wyodrębniają się cztery domeny komórkowe i dalej cztery organy somatycznego zarodka: liść, pęd, korzeń oraz stopka (Mikuła i in. 2015 b). Liniowy sposób różnicowania somatycznych zarodków *C. delgadii*, charakteryzujący się zachodzeniem podziałów komórkowych wyłącznie poprzecznych do osi eksplantatu oraz szeregowym układem podzielonych komórek, jest dotychczas nieopisanym w świecie roślin zjawiskiem biologicznym. Dowodzi to, że odmienny (od opisywanego w zygotycznej embriogenezie) wzór pierwszych podziałów kompetentnej komórki somatycznej nie jest przeszkodą w inicjowaniu SE i formowaniu somatycznych zarodków. Badania pokazały również, że fazie indukcji SE nie towarzyszą podziały komórek, które dają początek innym procesom morfogenetycznym.

Opracowany w 2015 roku system SE *C. delgadii* stał się punktem wyjścia do badań podjętych w niniejszej rozprawie doktorskiej. Niezależność od PGRs predestynuje ten obiekt eksperymentalny do badań nad endogenną hormonalną regulacją morfogenezy in vitro. Epidermalne, a w szczególności jednokomórkowe pochodzenie somatycznych zarodków sprawia, że *C. delgadii* stanowi dogodny model do badania interakcji i połączeń pomiędzy komórkami. Badania nad embriogenezą in vitro dla tego gatunku stanowią również doskonałe uzupełnienie dotychczasowej wiedzy w zakresie embriologii paproci.

5. Hipoteza badawcza i cel pracy

Paproć drzewiasta *C. delgadii* to gatunek, u którego etiolacja roślin donorowych i wycięcie eksplantatu są bodźcami wystarczającymi do zmiany dotychczasowego programu rozwojowego komórek i uruchomienia w nich procesu SE (Mikuła i in. 2015a, b). W niniejszej rozprawie doktorskiej postawiono więc hipotezę, że podstawą uzyskiwania embriogenicznego charakteru przez eksplantaty *C. delgadii* jest odpowiednia endogenna równowaga hormonalna, zaś wejście pojedynczych komórek somatycznych na drogę SE jest regulowane ograniczeniem symplastowej łączności pomiędzy komórkami eksplantatu.

Celem badań było poznanie i opisanie uwarunkowań związanych z nabywaniem embriogenicznego potencjału przez eksplantaty *C. delgadii* na poziomie struktury i ultrastruktury komórek, fizjologicznych zależności oraz symplastowej komunikacji.

W związku z powyższym określono:

- poziom endogennych hormonów i cukrów w eksplantatach zdolnych i niezdolnych do SE (Publikacja 1);
- dynamikę zmian w poziomie endogennych hormonów i cukrów w trakcie 14-dniowej kultury eksplantatów zdolnych do SE (Publikacja 1);
- wpływ inhibitorów transportu i biosyntezy hormonów na SE i syntezę wybranych hormonów (Publikacja 2);
- zmiany zachodzące w strukturze i ultrastrukturze komórek eksplantatów w czasie indukcji SE (Publikacje 3 i 4);
- zmiany w symplastowej komunikacji w czasie nabywania embriogenicznego potencjału i wczesnego etapu formowania somatycznych zarodków (Publikacja 3);
- wpływ czynników zależnych od cech fizycznych eksplantatu oraz egzogennych czynników stresowych na pochodzenie somatycznych zarodków i efektywność SE (Publikacja 4).

6. Materiały i metody

Rozdział ten podsumowuje metody badawcze wykorzystywane w doświadczeniach objętych niniejszą rozprawą doktorską i użyte do opisu SE u *C. delgadii*. Schemat przeprowadzonych badań z podziałem na cztery publikacje wchodzące w skład dysertacji przedstawiono na Rycinie 1.

Materiał roślinny wykorzystywany w trakcie badań stanowiły akseniczne kultury sporofitów *C. delgadii* wyprowadzone i utrzymywane w PAN Ogrodzie Botanicznym - CZRB w Powsinie. Źródłem eksplantatów były najmłodsze liście pochodzące z 2-5-liściowych sporofitów utrzymywanych w warunkach fotoperiodu (16/8 h światło/ciemność; nieetiolowane) lub w całkowitej ciemności (etiolowane). Analizom poddano eksplantaty ogonków liściowych i międzywęźli pochodzące z etiolowanych sporofitów oraz ogonków pobieranych z nieetiolowanych sporofitów. Kultury eksplantatów utrzymywano w ciemności. Wszystkie eksperymenty prowadzono na pożywce zawierającej połowę mikro- i makroelementów pożywki MS (Murashige i Skoog 1962) i pełny zestaw witamin ($\frac{1}{2}$MS) bez regulatorów wzrostu lub zawierającej inhibitory wzrostu i transportu hormonów.

Ocenę efektywności SE przeprowadzano po dwóch miesiącach kultury. Analizowano procent eksplantatów wytwarzających somatyczne zarodki, liczbę somatycznych zarodków na reagujący eksplantat oraz współczynnik zdolności do produkcji somatycznych zarodków.

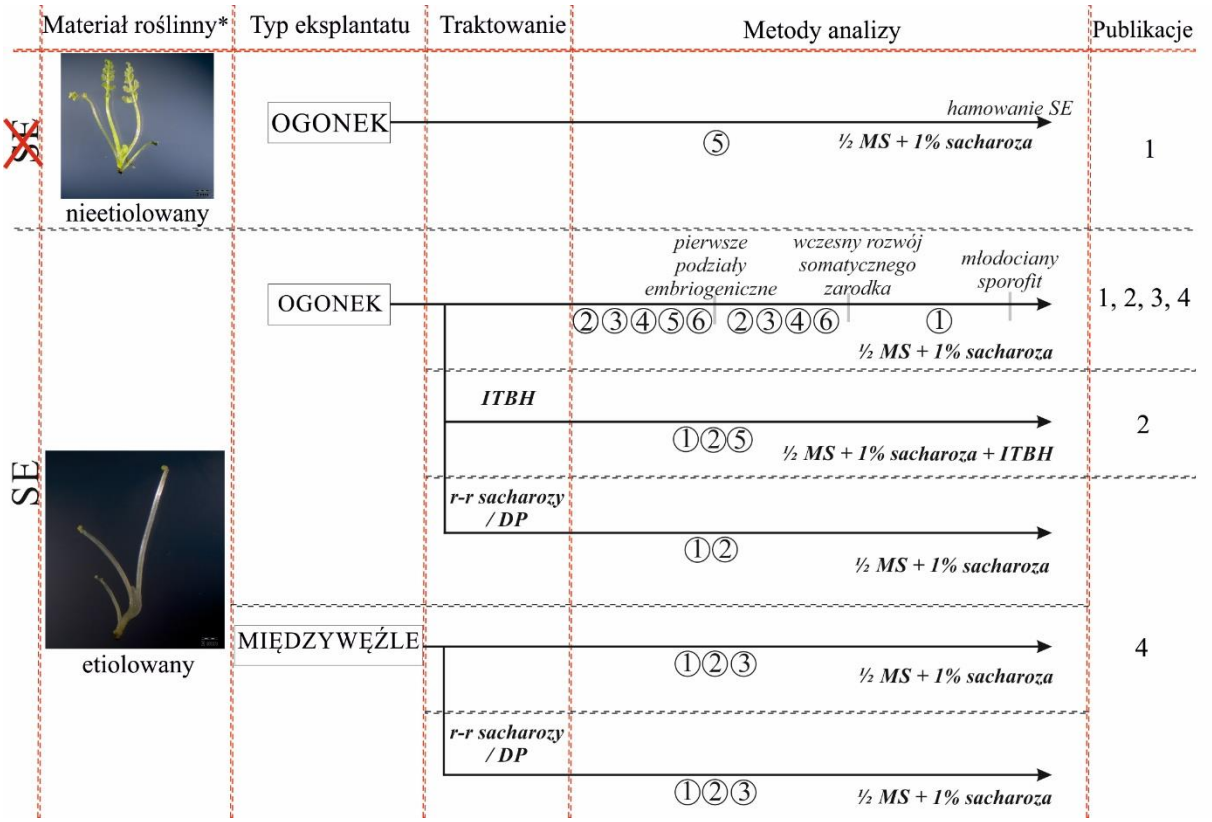
Do zobrazowania cytomorfologicznych zmian w eksplantatach w trakcie indukcji SE i rozwoju somatycznego zarodka wykorzystywano mikroskopię świetlną (analiza strukturalna), transmisyjną (TEM; analiza ultrastrukturalna) i środowiskową skaningową (ESEM; analiza morfologiczna) mikroskopię elektronową. Obserwacje w mikroskopie świetlnym i TEM prowadzono na materiale roślinnym utrwalonym w mieszaninie 2,5% glutaraldehydu i 2,5% formaldehydu, zatopionym w żywicy epoksydowej (Epon). Preparaty półcienkie (2 μm) i ultracienkie (90 nm) wykonano w Katedrze Botaniki Szkoły Głównej Gospodarstwa Wiejskiego (SGGW) w Warszawie, gdzie została również przeprowadzona analiza TEM. Obserwację w ESEM prowadzono przyżyciowo na nieutrwalanym materiale z wykorzystaniem mikroskopu środowiskowego w Centrum Analitycznym SGGW. Analizę w TEM o wysokiej rozdzielczości wykonano w Instytucie Nauk o Materiałach Uniwersytetu Śląskiego (UŚ) w Chorzowie.

Badania na poziomie fizjologicznym zostały przeprowadzone z wykorzystaniem wysokosprawnej chromatografii cieczowej (ang. high-performance liquid chromatography; HPLC) w Instytucie Fizjologii Roślin PAN w Krakowie. Zbadano zawartość hormonów tj. auksyny (IAA; kwas indolilo-3-octowy), siedmiu cytokinin (cis/trans zeatyna, rybozyd cis/trans zeatyny, kinetyna, rybozyd kinetyny, izopentenyloadenina) oraz kwasu abscysynowego (ABA). Określono również poziom endogennych cukrów: glukozy, fruktozy i sacharozy.

Badania symplastowej komunikacji przeprowadzano w oparciu o zastosowanie niskocząsteczkowych fluorochromów transportu symplastowego (tj. fluoresceiny i trójsodowej soli kwasu 8-hydroksypyreno-1,3,6-sulfonowego; HPTS). Eksplantaty traktowano fluorochromem przez 60 min i następnie płukano w wodzie demineralizowanej w celu usunięcia fluorochromu nieznajdującego się w komórkach. Żywotność eksplantatów oceniano poprzez barwienie jodkiem propidyny i błękitem Evans'a. Do analizy

rozmieszczenia fluorochromów wykorzystano laserowy skaningowy mikroskop konfokalny z wielopasmowym laserem argonowym do wzbudzania fluorescencji. Doświadczenia prowadzono w Instytucie Biologii, Biotechnologii i Ochrony Środowiska UŚ w Katowicach.

Uzyskane wyniki zostały poddane analizie statystycznej z wykorzystaniem oprogramowania Statgraphics Plus oraz STATISTICA wersja 6,0. Wyniki wyrażono jako średnie pochodzące z minimum 2 powtórzeń biologicznych oraz odchylenia standardowe. Zastosowano jednoczynnikową lub wieloczynnikową analizę wariancji wraz z testem najmniej znaczących różnic Fisher'a (Publikacja 1, 2, 4), oraz test T-studenta (Publikacja 1, 2). Poziom istotności wszystkich testów ustalono na 0,05.



Rycina 1. Schemat przeprowadzonych doświadczeń i wykorzystanych metod badawczych.
SE – somatyczna embriogeneza; ~~SE~~ – brak/hamowanie SE;
** – 3-5-liściowy sporofit rosnący w warunkach 16/8 h fotoperiodu (nieetiolowany) lub w ciemności (etiolowany); OGONEK – 2,5 mm fragment ogonka liściowego wycięty u podstawy najmłodszego liścia sporofitu; MIĘDZYWĘŹLE – fragment o różnej długości, znajdujący się pomiędzy najmłodszym a kolejnym liściem sporofitu;*
***ITBH** – inhibitory transportu i biosyntezy hormonów: kwas 2,3,5-trijodobenzoesowy (TIBA; 4, 8, 10, 12, 16, 20 i 30 μM), 1-metylo-3-fenylo-5- [3- (trifluorometylo) fenylo]-4-pirydynon (fluridon; 1, 10, 20, 30 i 40 μM) oraz kwas salicylowy (SA; 1, 5, 10, 25, 50, 75, 100 i 125 μM);*
***r-r sacharozy** – inkubacja inicjalnych eksplantatów w wodnych roztworach sacharozy (0,4; 0,5; 0,6 i 0,7 M) przez 15, 30, 45 lub 60 minut;*
***DP** – desykacja powietrzna w przepływie sterylnego powietrza przez 15, 30, 45 lub 60 minut;*
① – ocena efektywności SE;
② – analiza strukturalna; mikroskop świetlny;
③ – analiza morfologiczna; ESEM;
④ – analiza ultrastrukturalna; TEM;
⑤ – analiza fizjologiczna; HPLC;
⑥ – analiza symplastowej komunikacji.

7. Omówienie uzyskanych wyników

7.1. Publikacja 1

Grzyb M, Kalandyk A, Waligórski P, Mikuła A (2017) The content of endogenous hormones and sugars in the process of early somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb. Plant Cell Tissue and Organ Culture 129:387-397

SE u *C. delgadii,* indukowana niezależnie od egzogennych regulatorów wzrostu, jest doskonałym modelem do badań nad endogenną hormonalną regulacją morfogenezy in vitro. Dotychczas prowadzone eksperymenty nad tym gatunkiem wykazały, że etiolacja roślin donorowych jest warunkiem koniecznym do wprowadzenia materiału inicjalnego w „stan gotowości", zaś kluczowym czynnikiem wyzwalającym uruchomienie embriogenicznego potencjału jest wycięcie eksplantatu (Mikuła i in. 2015a). Celem pracy było poznanie wpływu etiolacji na poziom fitohormonów i cukrów obecnych w wyjściowym materiale roślinnym oraz określenie zmian w ich zawartości podczas indukcji SE i wczesnego etapu rozwoju somatycznych zarodków.

Do inicjacji kultury użyto 2,5 mm fragmenty ogonków liściowych pochodzące z etiolowanych i nieetiolowanych sporofitów. Poziom endogennych hormonów (tj. kwasu abscysynowego (ABA), kwasu indolilo-3-octowego (IAA), cytokinin (CK): cis/trans zeatyny, rybozydu cis/trans zeatyny, kinetyny, rybozydu kinetyny i izopentenyloadeniny) i cukrów (tj. glukozy, fruktozy i sacharozy) zmierzono z wykorzystaniem HPLC. Analizę chromatograficzną przeprowadzono dla inicjalnych eksplantatów: niezdolnych (pochodzących z nieetiolowanych sporofitów) i zdolnych (pochodzących z etiolowanych sporofitów) do SE, a także dla materiału roślinnego pobieranego co 2 dni w czasie 14-dniowej kultury indukującej SE.

W etiolowanym eksplantacie inicjalnym stwierdzono: 12-krotnie niższą zawartość ABA, znacznie niższy stosunek współczynników ABA/CK i ABA/IAA, oraz znacznie niższą zawartość wszystkich analizowanych cukrów niż w eksplantacie nieetiolowanym. Analiza etiolowanego eksplantatu poddawanego 14-dniowej kulturze wykazała, że jego wycięcie powoduje obniżenie poziomu wszystkich analizowanych hormonów, ale nie wpływa na równowagę pomiędzy nimi. W czasie kultury stosunki ABA/CK i ABA/IAA utrzymywały się na takim samym poziomie jak w eksplantacie inicjalnym. Zmianie ulegał jedynie stosunek IAA/CK. Poziom cytokinin, z wyjątkiem 6 dnia, przewyższał poziom IAA. Od 10 dnia kultury obserwowano wzrost poziomu rybozydu trans zeatyny. Ponadto, stwierdzono akumulację heksoz w 4-tym dniu kultury, a 2 dni później 12-krotny wzrost poziomu sacharozy.

Uzyskane wyniki dowodzą, że:

1) warunki świetlne, w których rozwijają się donorowe rośliny, wpływają znacząco na poziom i równowagę endogennych hormonów i cukrów;
2) nabycie kompetencji do SE przez komórki inicjalnego eksplantatu jest ściśle związane z etiolacją donorowych roślin, która powoduje obniżenie zawartości endogennego ABA i cukrów oraz podniesienie poziomu endogennych IAA i CK;
3) nagły wzrost poziomu endogennej sacharozy może być czynnikiem stresowym indukującym zmianę programu rozwojowego komórek somatycznych w eksplantatach *C. delgadii*;
4) wczesne etapy inicjowania somatycznych zarodków związane są z przewagą poziomu CK nad IAA, zwłaszcza rybozydu trans zeatyny, który może być zaangażowany w intensywne podziały komórkowe powstającego zarodka.

7.2. Publikacja 2

Grzyb M, Kalandyk A, Mikuła A (2018) Effect of TIBA, fluridone and salicylic acid on somatic embryogenesis and endogenous hormone and sugar contents in *Cyathea delgadii* Sternb. Acta Physiologiae Plantarum 40:1

Zawartość fitohormonów w komórkach eksplantatu inicjalnego i ich wzajemna równowaga warunkują reakcję w kulturze in vitro (Jimenez 2005; Fehér 2015). Celem badań było określenie wpływu inhibitorów transportu i biosyntezy hormonów na indukcję SE i formowanie somatycznych zarodków oraz ich wpływ na poziom endogennych hormonów w trakcie inicjalnej kultury eksplantatów ogonków liściowych *C. delgadii.*

2,5 mm fragmenty etiolowanych ogonków liściowych inkubowano na pożywkach zawierających inhibitory: 1) transportu auksyny (kwas 2,3,5-trijodobenzoesowy, TIBA; 4-30 μM), 2) biosyntezy ABA (fluridon; 1-40 μM) i 3) biosyntezy etylenu (kwas salicylowy; 1-125 μM). Po dwóch miesiącach kultury oceniano efektywność formowania somatycznych zarodków po zastosowaniu poszczególnych inhibitorów. Z wykorzystaniem HPLC przeanalizowano poziom endogennych hormonów i cukrów w materiale roślinnym pobieranym co 2 dni w czasie trwania 10 dniowej kultury.

Wykazano, że zastosowanie 30 μM TIBA lub 40 μM fluridonu lub 125 μM kwasu salicylowego całkowicie hamuje powstawanie somatycznych zarodków. Substancje te istotnie zmieniają poziom endogennego IAA i/lub ABA i/lub cytokinin, jak również modyfikują stosunki poszczególnych hormonów względem siebie. Zaburzenie poziomu fitohormonów skutkowało także zmianą zawartości endogennych cukrów (glukozy, fruktozy, sacharozy). TIBA i fluridon wpływały znacząco na zawartość IAA, ABA, CK i cukrów. Kwas salicylowy wpływał na zawartość IAA i cukrów, modyfikował również stosunek IAA/CK. Wszystkie inhibitory podnosiły poziom endogennej kinetyny.

Uzyskane wyniki dowodzą, że:

1) inhibitory wpływają nie tylko na hormony będące celem ich działania, ale także modyfikują zawartość pozostałych fitohormonów, jak również endogennych cukrów;
2) w wyniku traktowania inhibitorami zaburzone zostają równowaga hormonalna i zawartość cukrów w eksplantatach, co uniemożliwia przekierowanie programu rozwojowego komórek somatycznych w kierunku SE i hamuje tworzenie somatycznych zarodków.

7.3. Publikacja 3

Grzyb M, Wróbel-Marek J, Kurczyńska E, Sobczak M, Mikuła A (2020) Symplasmic isolation contributes to somatic embryo induction and development in the tree fern *Cyathea delgadii* Sternb. Plant and Cell Physiology. Doi: 10.1093/pcp/pcaa058

System cytoplazmatycznej łączności jest szybką i precyzyjną drogą wymiany informacji pomiędzy komórkami, za pomocą której kontrolowane są procesy morfogenezy roślin (Marzec i Kurczynska 2014; Otero i in. 2016). Epidermalne i jednokomórkowe pochodzenie somatycznych zarodków z eksplantatów ogonków liściowych *C. delgadii* predestynuje ten obiekt eksperymentalny do prowadzenia badań nad rolą symplastowej komunikacji w procesie SE. Celem niniejszej pracy było wykazanie zmian w łączności symplastowej komórek eksplantatu ogonka w czasie indukcji SE oraz we wczesnym etapie formowania się somatycznego zarodka paproci *C. delgadii*.

Do inicjacji kultury użyto 2,5 mm fragmenty ogonków liściowych pobierane z etiolowanych sporofitów. Analizy prowadzono w czasie 21-dniowej kultury. Eksplantaty inicjalne stanowiły kontrolę. Z wykorzystaniem mikroskopu konfokalnego prześledzono przemieszczanie się niskocząsteczkowych fluorochromów transportu symplastowego, tj. trójsodowej soli kwasu 8-hydroksypyreno-1,3,6-sulfonowego (HPTS) i fluoresceiny. Analizę cytomorfologiczną wykonano z wykorzystaniem mikroskopii świetlnej i transmisyjnej oraz środowiskowej skaningowej mikroskopii elektronowej.

Wykazano, że w inicjalnych eksplantatach ogonków liściowych badane fluorochromy były równomiernie rozprzestrzenione w epidermie oraz wnikały w głąb eksplantatu do 2-3 warstw komórek kory. W eksplantatach poddanych 6-cio dniowej kulturze dochodziło do ograniczenia ich przepływu pomiędzy niektórymi komórkami epidermy oraz pomiędzy epidermą a korą. Tuż przed wystąpieniem pierwszych podziałów dających początek somatycznym zarodkom zaobserwowano szereg zmian w ultrastrukturze komórek epidermalnych, polegających na ograniczeniu wielkości wakuol i zwiększeniu ich ilości, podniesieniu gęstości cytoplazmy czy odkładaniu się elektronowo gęstych substancji w ścianie komórkowej.

W czasie różnicowania somatycznych zarodków stwierdzono ograniczenia w komunikacji symplastowej pomiędzy zarodkiem a komórkami eksplantatu. Somatyczny zarodek, do etapu trzykomórkowej struktury, stanowił pojedynczą domenę symplastową, w której fluorochromy przemieszczały się swobodnie. Dalsze podziały w obrębie zarodka skutkowały ograniczeniem przepływu fluorochromu pomiędzy jego komórkami, czego efektem było formowanie czterech domen symplastowych odpowiadających czterem segmentom ciała zarodka.

Uzyskane wyniki dowodzą, że:

1) warunkiem wyzwolenia embriogenicznej kompetencji jest ograniczenie symplastowej komunikacji pomiędzy komórkami eksplantatu skorelowanej z przebudowaniem ich cytoplazmy;
2) symplastowa komunikacja odgrywa kluczową rolę w rozwoju somatycznego zarodka, natomiast jej ograniczenie w relacji pomiędzy poszczególnymi czterema domenami prowadzi do różnicowania organów.

7.4. Publikacja 4

Grzyb M, Mikuła A (2019) Explant type and stress treatment determine the uni- and multicellular origin of somatic embryos in the tree fern *Cyathea delgadii* Sternb. Plant Cell, Tissue and Organ Culture 136:221–230

Materiał donorowy będący źródłem inicjalnych eksplantatów oraz stres to jedne z kluczowych czynników wpływających na odpowiedź morfogenetyczną roślin nasiennych w warunkach kultury in vitro (Gaj, 2004; Loyola-Vargas and Ochoa-Alejo 2016). Z uwagi na niewielką liczbę przebadanych pod kątem SE gatunków roślin zarodnikowych, czynniki, które odpowiadają za jej indukcję, są w tej grupie roślin słabo poznane. W pracy analizowano więc wpływ rodzaju eksplantatu, długości i średnicy donorowego liścia, traktowania roztworami sacharozy i desykacji powietrznej na pochodzenie somatycznych zarodków i efektywność SE u paproci *C. delgadii*.

Do inicjacji kultury użyto 2,5 mm fragmenty ogonków liściowych (wycięte z najmłodszego liścia długości 5, 10, 15 lub 20 mm i średnicy <300 lub >300 μm) i fragmenty międzywęźli (długości 0,5; 1,0; 1,5; 2,0 lub 2,5 mm) izolowane z etiolowanych sporofitów. Część eksplantatów przed wyłożeniem na pożywkę poddawano desykacji powietrznej (w przepływie sterylnego powietrza przez 15, 30, 45 lub 60 min.) bądź traktowano roztworami sacharozy (0,4; 0,5; 0,6 i 0,7 M przez 15, 30, 45 i 60 min.). Wyniki wizualizowano z wykorzystaniem mikroskopii świetlnej i środowiskowej skaningowej mikroskopii elektronowej.

Najwyższą efektywność SE otrzymano na eksplantacie ogonka liściowego, który pobrano z liścia długości 10 mm i średnicy mniejszej niż 300 μm. Somatyczne zarodki otrzymane na eksplantacie ogonka były inicjowane podziałami pojedynczych komórek epidermy, zaś w przypadku eksplantatów międzywęźli powstawały poprzez jednoczesne podziały wielu sąsiadujących komórek epidermy i kory pierwotnej. Mimo iż podziały komórkowe zachodziły intensywniej na międzywęźlach, to prowadziły one do uformowania średnio zaledwie 3 zarodków. Desykacja powietrzna hamowała SE na eksplantatach ogonków i nieznacznie podnosiła liczbę somatycznych zarodków powstających na eksplantatach międzywęźli (do 4 sztuk). Traktowanie roztworem sacharozy hamowało SE na eksplantatach ogonków, zaś w przypadku międzywęźli powodowało nawet 4-krotne podniesienie jej efektywności. Analiza mikroskopowa wykazała, że zwiększenie efektywności procesu SE uzyskiwanego na międzywęźlach zachodzi poprzez zmianę drogi różnicowania somatycznych zarodków z wielo- w jednokomórkową.

Uzyskane wyniki dowodzą, że:

1) materiał donorowy będący źródłem eksplantatów istotnie wpływa na efektywność SE;
2) pochodzenie somatycznych zarodków *C. delgadii* jest skorelowane z typem eksplantatu użytego do inicjacji kultury;
3) drogą różnicowania somatycznych zarodków na eksplantatach międzywęźli można sterować za pomocą egzogennego stresu osmotycznego.

8. Podsumowanie badań

Chociaż proces SE jest znany od ponad 60 lat, to badania prezentowane w niniejszej rozprawie doktorskiej są pionierskie dla paproci, kladu Monilofita oraz grupy roślin zarodnikowych. Ponadto ich skoncentrowanie na precyzyjnie określonej fazie indukcji, w której somatyczne komórki uzyskują embriogeniczny charakter, oraz bardzo wczesnej fazie różnicowania somatycznych zarodków sprawia, że otrzymane wyniki wnoszą również istotny wkład w poznanie najbardziej fascynującego badaczy wczesnego etapu SE.

Prowadzenie badań z użyciem modelu eksperymentalnego niewymagającego wykorzystania PGRs pozwoliło na precyzyjne zbadanie i opisanie po raz pierwszy na świecie przemian endogennych hormonów i cukrów zachodzących w fazie indukcji i wczesnym etapie fazy ekspresji bezpośredniej SE. Uzyskany obraz odpowiedzi morfogenetycznej eksplantatu, wywoływanej jedynie jego wycięciem z rośliny donorowej, rzuca nowe światło na reakcję jego komórek. Warto podkreślić, że wycięcie eksplantatu poprzedza inicjację większości kultur roślinnych, jednakże reakcja na ten stres jest sporadycznie brana pod uwagę w badaniu podstaw SE. Podjęta w rozprawie szeroka analiza z wykorzystaniem inhibitorów biosyntezy i transportu hormonów wykazała skomplikowane zależności i interakcje pomiędzy różnymi klasami hormonów i cukrami, dowodząc, że zaburzona endogenna równowaga tych związków w eksplantatach uniemożliwia przekierowanie programu rozwojowego komórek somatycznych w kierunku SE. Prezentowane wyniki po raz pierwszy przedstawiają także analizę zmian w komunikacji symplastowej w SE paproci i dowodzą, że również w grupie roślin zarodnikowych łączność pomiędzy komórkami zaangażowanymi w tranzycję embriogeniczną jest niezwykle istotna. Model eksperymentalny *C. delgadii* bazujący na jednokomórkowym, epidermalnym pochodzeniu zarodków pozwolił na zbadanie symplastowej komunikacji na bardzo wczesnym etapie SE i prześledzenie przemieszczania fluorochromów już w kilkukomórkowym somatycznym zarodku, co dotychczas (ze względu na brak odpowiedniego systemu eksperymentalnego) było praktycznie niemożliwe. Uzyskana wiedza w zakresie czynników indukujących SE, w tym rola etiolacji roślin donorowych, może poszerzyć praktyczne wykorzystanie tego procesu np. u roślin, u których zazwyczaj ta wysoce efektywna droga regeneracji jest trudna do zainicjowania.

Uzyskane rezultaty pozwoliły na:

1. wykazanie, że równowaga hormonalna w inicjalnym etiolowanym eksplantacie pobranym z donorowej rośliny jest podstawą uzyskiwania przez jego komórki embriogenicznych zdolności, zaś uruchomienie programu SE jest ściśle związane z wycięciem eksplantatu i następującymi później drastycznymi zmianami w poziomie endogennej sacharozy;
2. udokumentowanie, że ograniczenie symplastowej komunikacji jest jednym z pierwszych objawów nabywania embriogenicznej kompetencji i kluczowym warunkiem rozwoju somatycznego zarodka;
3. opracowanie nowego systemu eksperymentalnego *C. delgadii* bazującego na bezpośrednim, wielokomórkowym pochodzeniu somatycznych zarodków z eksplantatu

międzywęźla; oraz metody sterowania jego efektywnością poprzez modyfikowanie drogi inicjowania zarodków;
4. pogłębienie wiedzy na temat SE paproci oraz paprociowego systemu modelowego;
5. wykazanie, że system eksperymentalny *C. delgadii* może być z powodzeniem wykorzystywany w uzupełnianiu podstawowej wiedzy na temat procesu SE.

Potwierdzono tym samym hipotezę, że podstawą uzyskiwania embriogenicznego charakteru przez eksplantaty *C. delgadii* jest odpowiednia endogenna równowaga hormonalna, zaś wejście pojedynczych komórek somatycznych na drogę SE jest regulowane ograniczeniem symplastowej łączności pomiędzy komórkami eksplantatu.

9. Dalsze plany badawcze

Dwa niezależne modele indukowania SE u *C. delgadii* (z pojedynczych komórek eksplantatu ogonka lub z wielu komórek eksplantatu międzywęźla) oraz możliwość manipulowania drogą powstawania somatycznych zarodków, tworzą doskonałą platformę badań nad poznaniem mechanizmu indukowania SE. Dalsze eksperymenty będą skupione na określeniu podstaw odmiennej drogi różnicowania somatycznych zarodków, mającej swoje źródło w eksplantatach ogonków i międzywęźli. Inspiracją do przeprowadzenia wieloaspektowych badań porównawczych obu eksplantatów jest chęć udowodnienia tezy postawionej w 1985 przez Maheswarana i Williams mówiącej, iż droga różnicowania somatycznych zarodków zależy od wieku eksplantatu inicjalnego i zdolności jego komórek do wzajemnych interakcji.

Z uwagi na wykazany kluczowy udział symplastowej komunikacji w indukowaniu SE i rozwoju somatycznego zarodka, badania zostaną pogłębione o analizę morfologii i składu ściany komórkowej. Zakłada się bowiem, że zmiany właściwości ściany komórkowej w czasie inicjowania SE mogą być markerem różnicowania komórek. Szczególna uwaga będzie zwrócona na udokumentowanie obecności i rozmieszczenia plasmodesm w ścianach komórkowych eksplantatu, a także na ocenę ich drożności i biologicznej aktywności. W celu jakościowego określenia zmian przeprowadzona zostanie analiza immunohistochemiczna składników budujących ściany komórek eksplantatów inicjalnych, a także w trakcie ich kultury in vitro. Analiza powyższych parametrów pozwoli na wyznakowanie w epidermie eksplantatu komórek zdolnych i niezdolnych do SE, zanim pojawią się pierwsze podziały komórkowe dające początek formowaniu somatycznych zarodków. Tak wyznakowane komórki będą mogły zostać poddane precyzyjnym analizom porównawczym. Dlatego kolejnym krokiem, który zostanie podjęty będzie analiza transkryptomu nastawiona na identyfikację genów ulegających zróżnicowanej ekspresji w pojedynczych, embriogenicznych i nieembriogenicznych komórkach eksplantatu. Takie podejście umożliwi śledzenie niezakłóconej egzogennymi regulatorami wzrostu reorganizacji transkryptomu w czasie tranzycji somatycznych komórek roślinnych do stanu embriogenicznego.

Szerokie spektrum planowanych badań wykorzystujących unikatowy charakter opracowanych u *C. delgadii* modeli indukowania SE, może przynieść w przyszłości

odpowiedź na pytanie, jak pojedyncza komórka somatyczna zmienia swój program rozwojowy i staje się kompletną rośliną. Badania te otworzą również możliwość śledzenia ewolucji genów zaangażowanych w specyficzny dla świata roślin proces SE.

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11. Załączniki

Załącznik 1 – Publikacja 1

Załącznik 2 – Publikacja 2

Załącznik 3 – Publikacja 3

Załącznik 4 – Publikacja 4

Załącznik 5 – Oświadczenie doktorantki

Załącznik 6 –Oświadczenia współautorów

Plant Cell Tiss Organ Cult (2017) 129:387–397
DOI 10.1007/s11240-017-1185-8

ORIGINAL ARTICLE

The content of endogenous hormones and sugars in the process of early somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb.

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Received: 30 November 2016 / Accepted: 31 January 2017 / Published online: 15 February 2017

Abstract Somatic embryogenesis (SE) of *Cyathea delgadii* presents a model system for investigating the mechanisms associated with the acquisition of embryogenic competence by single epidermal cells of stipe explants cultured on plant growth regulator-free medium. The present work reveals relationship between endogenous hormone and sugar content in the process of early SE in *C. delgadii*. By comparing two types of initial explants, i.e. incapable (non-etiolated) and capable (etiolated) of SE, it was established that in etiolated explants, the glucose, fructose, sucrose, and abscisic acid (ABA) contents diminished, but indole-3-acetic acid (IAA) and cytokinins (CKs; i.e. *cis*/*trans* zeatin, *cis*/*trans*-zeatin riboside, kinetin, kinetin riboside, isopentenyladenosine) contents increased. The ratios between phytohormones revealed that a high concentration of ABA is the main factor inhibiting SE induction. Because of explant excision, a dramatic reduction in concentration of all phytohormones studied was observed, but hormonal balance and sugar content remained almost unchanged. During the 14-day-long culture, the ABA/CKs and ABA/IAA ratios remained constant, whereas the greatest differences were detected for the IAA/CKs and Z-type/iPA cytokinin ratios. Excluding day 6 of culture, cytokinins were found to be the predominant phytohormones over IAA. An almost 12-fold increase in soluble sucrose concentration at day 6 of culture might be the switch to the SE expression phase. Frequent cell divisions leading to somatic embryo formation are clearly associated with increase in t*rans*-zeatin riboside content.

Keywords Etiolation · Explant excision · High-performance liquid chromatography · PGR-free medium · Phytohormones

Abbreviations

ABA	Abscisic acid
c/t-Z	*Cis*/*trans* zeatin
c/t-ZR	*Cis*/*trans*-zeatin riboside
CKs	Total concentration of cytokinins
FW	Fresh weight
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
iPA	Isopentenyladenosine
Kin	Kinetin
KinR	Kinetin riboside
PGR	Plant growth regulator
SE	Somatic embryogenesis

Electronic supplementary material The online version of this article (doi:10.1007/s11240-017-1185-8) contains supplementary material, which is available to authorized users.

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Introduction

Somatic embryogenesis (SE) is an important pathway for the regeneration of plants, as well as an attractive system for the study of the morphology, physiology, and genetic mechanisms of embryo induction and development (Elhiti et al. 2013; Fehér 2015). Although this process was first described many years ago, our present knowledge about the acquisition of embryogenic competence by somatic cells remains unknown, and it is unexplored in ferns.

Understanding of the physiological mechanism that underlies the induction of SE is essential for the regulation of this process. Phytohormones are employed as signalling molecules for the induction of SE by mediating the signal transduction cascade leading to the reprogramming of gene expression patterns (Elhiti et al. 2013). Some hormones, such as abscisic acid (ABA) and cytokinins have been shown to help regulate the sugar metabolism and transport. On the other hand, sugars regulate the synthesis, conjugation and transport of phytohormones (for example ABA), and also the expression of components of phytohormone-response pathways (Gibson 2004). A direct link between sugar signalling and hormone biosynthesis has been described by Eveland and Jackson (2011). At present, it is not surprising that the availability of sugars in the induction medium helps regulate developmental processes in ferns (Mikuła et al. 2015a). However, the changes in endogenous sugar contents during SE induction have not yet been studied.

The relationships between endogenous hormone contents and SE have been the subject of many studies. However, most of them are limited to the comparisons of the non-embryogenic and embryogenic callus (Jiménez and Bangerth 2000; Pintos et al. 2002; Jiménez et al. 2005), the non-embryogenic explants and those with somatic embryos developed on the explant surface (Pérez-Jiménez et al. 2013), or the genotypes/explants with different embryogenic capability (Wenck et al. 1988; Centeno et al. 1997; Limanton-Grevet et al. 2000; Jiménez and Bangerth 2001a). Only a few reports on the progressive hormonal changes that occur during SE are available, but they differ in the type of explant, method (plant growth regulators or other stress factors) and manner of somatic embryo induction (i.e. direct or indirect), and culture conditions (photoperiodic or darkness) employed (Grieb et al. 1997; Charrière et al. 1999; Jiménez and Bangerth 2001b; Yang et al. 2012; Ayil-Gutiérrez et al. 2013). In these studies, the dynamic changes in the content of indole-3-acetic acid (IAA) (Ayil-Gutiérrez et al. 2013), or IAA and ABA and cytokinins (Grieb et al. 1997; Charrière et al. 1999), and also in gibberellin concentrations (Jiménez and Bangerth 2001a) during the induction of SE were described for *Coffea canephora* Pierre ex Froehner, *Daucus carota* L., *Helianthus annuus* L., and *Zea mays* L., respectively. Because the ratio of endogenous hormones controls a mode of morphogenetic response, the ratio of IAA or ABA to cytokinins (Żur et al. 2015), or the ratio of ABA to IAA (Centeno et al. 1997), or of various types of cytokinins (e.g. zeatin (Z)-type and isopentenyl (iP)-type; Grieb et al. 1997; Pintos et al. 2002) tend to be good indicators of the embryogenic competence of explants.

In plant material, endogenous hormone contents may be affected by many factors. For example, exogenously applied plant growth regulators (PGRs) can interact with phytohormones and change their concentrations (Ayil-Gutiérrez et al. 2013). Another factor causing hormonal balance disorders is explant excision (Thornburg and Li 1991; Yang et al. 2012). It was showed that wounding is the first event that provides signals for triggering the entire regenerative process (Iwase et al. 2011; Chen et al. 2016). Although excision is a commonly used procedure for the introduction of explants into *in vitro* culture, little attention has been paid to this treatment so far. Phytohormone concentrations and their signalling pathways can also be regulated by changes in the photoperiodic conditions (Symons and Reid 2003; Pacholczak et al. 2005). As a result of the light deficit, plants show a dramatic elongation of the stem (Suzuki and Kerbauy 2006). The plant response is often linked to reduced leaf development, enhanced apical dominance, and a re-allocation of resources from storage-favouring shoot elongation to reproductive growth (Suzuki and Kerbauy 2006; Mikuła et al. 2015b). Dark treatment of donor plantlets was reported to favour SE in *Helianthus annuus* L. (Fiore et al. 1997), *Dendranthema grandiflora* Tzvelev. (May and Trigiano 1991) and *Cyclamen persicum* Mill. (Takamura and Tanaka 1996). Moreover, Pacholczak et al. (2005) discovered a correlation between light intensity and total soluble sugars and endogenous ABA and IAA contents.

By culturing stipe explants of the tree fern *Cyathea delgadii* Sternb. on PGR-free half-strength Murashige and Skoog's (1/2MS) medium, in darkness, we established a unique model system for the study of SE (Mikuła et al. 2015b). In this system, somatic embryos regenerate directly from the single epidermal cells of stipe explants. However, the capacity of *C. delgadii* explants to undergo SE depends on the prevailing photoperiodic conditions during the development of plantlets (Mikuła et al. 2015a). Explants excised from sporophytes that developed under 16/8 photoperiod conditions are incapable of SE. In contrast, in etiolated explants, the first cell divisions typically starts at 10th day of culture, and during the next 4–6 days, many somatic embryos at linear developmental stage are observed (Mikuła et al. 2015b). The present study was undertaken to find out if the etiolation, which is a key factor in the capacity of stipe explants to form somatic embryos, has an effect

on the content and balance of hormones and sugars. The study was also designed to determine possible relationships between the concentration and balance of above-mentioned compounds in etiolated explants during their 14-day-long culture on PGR-free medium.

Materials and methods

Plant material and culture initiation

Embryogenic culture of *C. delgadii* was established following the procedure described earlier by Mikuła et al. (2015b). The somatic embryo-derived sporophytes were cultured on PGR-free medium containing half-strength Murashige and Skoog's (Murashige and Skoog 1962) macro- and micro-nutrients and full complement of vitamins (1/2MS) and 2% (w/v) sucrose, solidified with 0.7% plant agar (Duchefa Biochemie); pH 5.8. The cultures were maintained under the photoperiodic conditions (16/8 light–dark regime; 50 µM m^{-2} s^{-1}; defined as non-etiolated) or in constant darkness (defined as etiolated) for 5 months.

In experiments described here, sporophytes that had developed 4 or 5 leaves were used as a source of explants. The stipe explants measuring 2.5 mm in length were excised from the first frond of non-etiolated and etiolated sporophytes (Mikuła et al. 2015a) and used for high-performance liquid chromatography (HPLC) analysis. Moreover, etiolated explants were cultured on 1/2MS induction medium supplemented with 1% (w/v) sucrose, in constant darkness for 14 days. The plant material was collected for HPLC analysis every 2 days.

Samples preparation for HPLC analysis

Excised explants were immediately frozen in liquid nitrogen. The plant samples (about 80 explants per sample; 35 mg of FW) were lyophilised and homogenised in an ice-cold mixture of methanol, water, and formic acid (15/4/1) according to Dobrev and Kamínek (2002). An internal isotopic standard mixture consisting of deuterated IAA and ABA, and kinetin labelled with nitrogen ^{15}N was added to each sample during homogenisation. The prepared extract was fractionated using solid phase extraction (SPE) columns Oasis MCX (Waters), and three fractions were collected: "acidic" eluted with methanol for IAA and ABA analyses according to HPLC method of Štefančič et al. (2007), "basic" eluted with methanolic solution of ammonia for cytokinins analyses according to Żur et al. (2015), and "flow through" for carbohydrates analyses according to Hura et al. (2016).

Quantification of IAA and ABA

After IAA and ABA elution from SPE column by pure methanol, each sample was evaporated to dryness and reconstituted in 50 µl methanol (Żur et al. 2015). Finally, samples were analysed using a Supelco Ascentis RP-Amide column (7.5 cm×2.1 mm, 2.7 µm). Mobile phases consisted of 0.1% formic acid solution in water (solvent A) and acetonitrile:methanol (1:1) mixture (solvent B). Analyses were performed using gradient elution at a flow rate of 1.5 ml min^{-1} with gradient starting from 20% B at 0 min, rising to 80% B at 3 min, and back to 20% at 3.5 min, total analysis time was 4 min. The HPLC apparatus was Agilent Technologies 1290 Infinity equipped with Agilent Technologies 6460 Triple Quad LC/MS with Jet Stream. Capillary voltage was set to 4000 V, two the most abundant product ions were monitored using MRM (Multiple Reaction Monitoring) mode for each analysed compound. The most abundant product ion was used for quantification (quantifier), whereas the other ion was used to confirm the identity of the phytohormones (qualifier). The MRMs were set as follows in Supplementary Table 1. Agilent Technologies Mass Hunter 5 software was used for apparatus control, data collection, and processing.

Quantification of cytokinins

Cytokinins, such as t-Z, c-Z, t-ZR, c-ZR, Kin, KinR and iPA, were separated from the samples. Their sum was defined as the total content of cytokinins and indicated by the abbreviation CKs. For their structural similarity, cytokinins were categorised into Z-type (i.e. t-Z + c-Z + t-ZR + c-ZR).

Fractions of cytokinins were flushed out from the SPE column after collecting IAA and ABA. Impurities were first washed out with 0.35 M ammonia in water and then cytokinins were eluted with 0.35 M ammonia in 60% methanol (Żur et al. 2015). The collected fraction was evaporated to dryness, reconstituted in 50 µl methanol, and analysed using the same chromatographic system and HPLC column as described above. The solvent system consisted of water with 0.001% acetic acid (solvent A) and acetonitrile with 0.001% acetic acid (solvent B) at a flow rate of 1.5 ml min^{-1}, gradient profile was 2.5% B to 1 min, 10% B at 3 min, 25% B at 6 min, 75% B at 8 min and 2.5% B at 8.5 min. Capillary voltage was set to 4000 V, two the most abundant positive secondary ions were monitored as follows in Supplementary Table 2.

Quantification of soluble sugars

The "flow through" fraction collected from the SPE column was lyophilised and reconstituted according to the

procedure described by Janeczko et al. (2010). Samples were analysed on Agilent Technologies 1200 HPLC equipped with an amperometric detector ESA Coulochem II Analytical Cell 5040 with gold electrode. The Hamilton RCX-10 250×4.1 mm (Hamilton, Reno, NV, USA) HPLC column was used. The mobile phase comprised 100 mM sodium hydroxide solution in water, and the flow rate was set to 1.5 ml min^{-1}. The detector was set at an analytical potential of 200 mV, an oxidising potential of 800 m, and a reducing potential of −900 mV (with reference to a palladium electrode). Agilent Technologies ChemStation B04 software was used for apparatus control, data collection, and processing.

Statistical analysis

Data for endogenous hormone and sugar contents are presented as mean ± SD for three replicates (35 mg FW, i.e. about 80 explants, was considered as one replicate). Statistical analysis for etiolated and non-etiolated explants was performed using the Student's t test embedded in microsoft excel. Only a return of $p < 0.05$ was designated as being statistically significant. As the number of data for analysis conducted during the 14 days of culture was limited it was not possible to execute normality tests, thus a non-parametric Analysis of Variance (Kruskal Wallis test) was applied to calculate the significant differences between the comparisons (corrected $p \leq 0.05$). The parameters that contributed significantly to the final score were determined by Multiple comparisons of mean ranks. All statistical analyses were performed using STATISTICA version 6.0 (Stat Soft Inc., USA, 2001) package.

Results

The effect of etiolation on endogenous hormone and soluble sugar contents in initial explants

The concentration of phytohormones and soluble sugars in stipe explants derived from sporophytes developed under photoperiodic conditions (non-etiolated) and in those developed in darkness (etiolated) was significantly different (Fig. 1). Etiolated explants (capable of SE) were characterised by almost 12-fold lower concentration of ABA, 2-fold greater IAA content and 9.4-fold greater concentration of total cytokinins in comparison with non-etiolated explants (incapable of SE; Fig. 1a, b). Moreover, the content of some individual cytokinins such as KinR and c-ZR was more than 450 times greater in etiolated explants than in non-etiolated ones. The concentrations of hexoses and sucrose were more than 2- and 39-fold lower in etiolated explants than in the plant material kept under photoperiodic conditions, respectively (Fig. 1b).

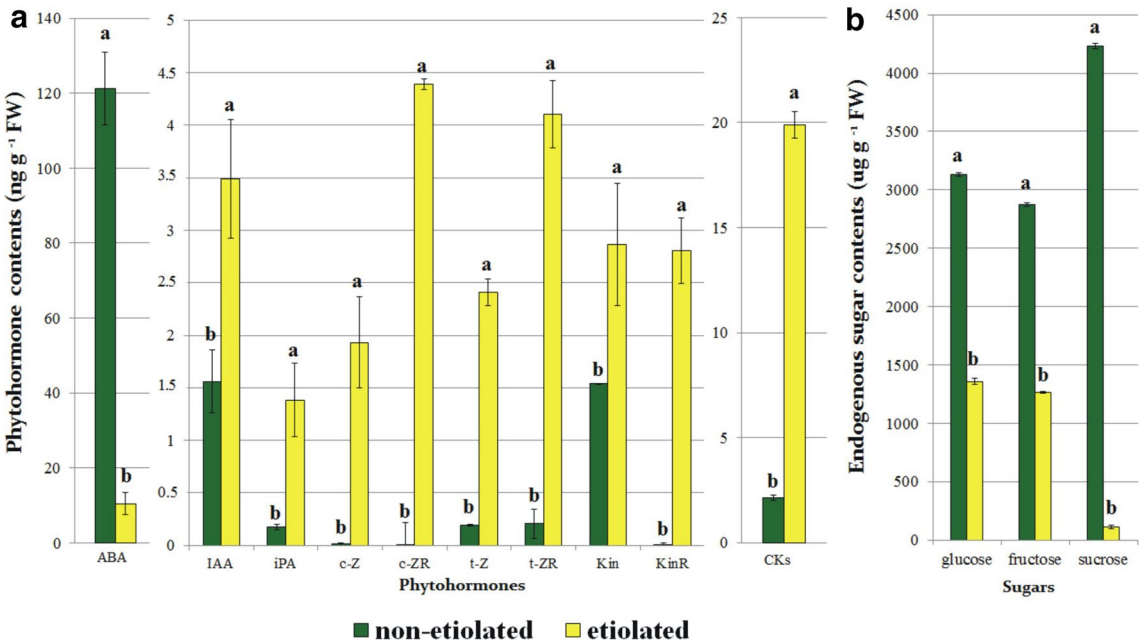

Fig. 1 Contents of **a** endogenous hormones and **b** soluble sugars in non-etiolated (incapable of somatic embryogenesis) and etiolated (capable of somatic embryogenesis) explants of *C. delgadii*. Data are the means (±SD) of three replicates. The Student's *t* test was used independently for each type of phytohormones and sugars. Mean values marked with the *same letter* do not differ significantly at the 0.05 confidence level. *ABA* abscisic acid, *CKs* the sum of c-Z, c-ZR, iPA, Kin, KinR, t-Z and t-ZR concentrations, *c-Z* cis zeatin, *c-ZR* cis zeatin riboside, *IAA* indole-3-acetic acid, *iPA* isopentenyladenosine, *Kin* kinetin, *KinR* kinetin riboside, *t-Z* trans zeatin, *t-ZR* trans zeatin riboside

In order to assess the hormonal balance in both types of plant material, the interaction between endogenous hormones was expressed by their ratios (Table 1). In non-etiolated explants, the ratios of IAA and ABA to individual cytokinins (also to CKs), as well as ABA to IAA, were much greater than those in etiolated ones. This was especially evident in the case of KinR, c-ZR and c-Z. Etiolation of donor plantlets resulted in equalisation of all ratios mentioned above (excluding IAA/Kin). Only in case of the ratio of Z-type cytokinin to iPA, its value was lower in non-etiolated explants (2.4) than in etiolated explants (9.3) (Table 1).

Changes in endogenous hormone and soluble sugar contents during 14-day-long culture

To characterise the physiological changes that occur during early SE in etiolated explant of *C. delgadii*, the content of endogenous hormones (Fig. 2a–e) and sugars (Fig. 2f) was determined during 14 days of culture. The content of all phytohormones studied diminished rapidly after excision of explant (i.e. at day 2 of culture). Between days 4 and 14, their concentrations remained relatively stable, excluding IAA, whose concentration decreased at day 10 (Fig. 2b), and Kin, whose concentration increased at day 12 (Fig. 2e), and t-ZR, whose amount increased starting on day 8 of culture (Fig. 2d).

The hormonal balance during induction of SE is presented in Table 2. Results showed that the ratios of IAA to the individual cytokinins (as well as to CKs) increased reaching its maximum at day 6 of culture. The ratio of Z-type cytokinin to iPA was significantly lower between days 2 and 8 of culture. After that, its value increased gradually. The values of the ABA/CKs (as well as to individual cytokinins) and ABA/IAA ratios remained almost unchanged during 14-day-long culture of stipe explants (Table 2).

The explant excision did not influence significantly the content of endogenous sugars (Fig. 2f). Intensive sucrose accumulation (with a mean 12-fold increase) was observed at day 6 of culture. Over the next 2 days, the level diminished by 37%, and remained relatively stable up to day 14. An increase in the hexose concentrations was observed at day 4. Then, content of both the glucose and fructose gradually diminished.

Table 1 Ratios of different phytohormones in non-etiolated and etiolated initial explants of *C. delgadii*

Phytohormone ratios	Non-etiolated explants	Etiolated explants	Non-etiolated/etiolated explants
IAA/CKs	**0.73 ± 0.1a**	**0.18 ± 0.1b**	**4.0**
IAA/t-Z	8.1 ± 2.1a	1.5 ± 0.7b	5.4
IAA/c-Z	145.1 ± 13.9a	1.8 ± 1.1b	80.6
IAA/t-ZR	7.6 ± 2.8a	0.9 ± 0.5b	8.4
IAA/c-ZR	159.6 ± 6.9a	0.8 ± 0.5b	199.5
IAA/Kin	1.0 ± 0.1a	1.2 ± 0.2b	0.8
IAA/KinR	435.3 ± 12.4a	1.2 ± 0.5b	362.8
IAA/iPA	9.0 ± 1.9a	2.5 ± 0.5b	3.6
ABA/CKs	**56.8 ± 3.2a**	**0.53 ± 0.2b**	**107.2**
ABA/t-Z	626.3 ± 44.1a	4.3 ± 1.9b	145.7
ABA/c-Z	11,275.9 ± 63.1a	5.4 ± 1.6b	2088.1
ABA/t-ZR	591.8 ± 16.6a	2.6 ± 0.7b	227.6
ABA/c-ZR	12,400.5 ± 24.1a	2.4 ± 0.6b	5166.9
ABA/Kin	78.9 ± 9.7a	3.7 ± 2.0b	21.3
ABA/KinR	33,829.6 ± 80.6a	3.7 ± 1.8b	9143.1
ABA/iPA	695.8 ± 24.2a	7.6 ± 3.0b	91.6
ABA/IAA	**77.7 ± 12.0a**	**2.7 ± 0.4b**	**28.8**
Z-type/iP-type	**2.4 ± 0.4a**	**9.3 ± 4.9b**	**0.3**

Data are the means (±SD) of three replicates. The Student's t test was used independently for each type of ratio determined. Mean values marked with the same letter do not differ significantly at the 0.05 confidence level

Values marked with the same letter differ insignificantly at the 0.05 confidence level according to Student's *t* test

ABA abscisic acid, *CKs* the sum of c-Z, c-ZR, Kin, KinR, t-Z, t-ZR, and iPA concentrations, *c-Z* cis zeatin, *c-ZR* cis zeatin riboside, *IAA* indole-3-acetic acid, *iPA* isopentenyladenosine, *Kin* kinetin, *KinR* kinetin riboside, *t-Z* trans zeatin, *t-ZR* trans zeatin riboside, *Z-type* the sum of c-Z, c-ZR, t-Z, t-ZR concentrations

Discussion

The morphogenetic response of plant material *in vitro* is greatly influenced by the ontogenetic age of the tissue or organ which is used as the source of the initial explant (Gaj 2004). Along with changes in the degree of tissue differentiation, the physiological state of cells is modified. The basis of these changes is an altered pattern of endogenous hormone synthesis, conjugation and/or inactivation, which can be modulated by the use of many stress-related factors (Fehér 2015) as well as by etiolation and explant excision.

Etiolation resulting in altered concentration and balance of endogenous hormones

Light affects the biosynthesis and signalling of endogenous hormones (Symons and Reid 2003). In cryptogamic plants, light is a crucial factor in controlling the regeneration pathways, including SE (Mikuła et al. 2015a). Therefore, we carried out quantitative analysis of ABA, IAA and cytokinin contents in etiolated (capable of SE) and non-etiolated (incapable of SE) stipe explants of *C. delgadii*, and found extreme differences in both the hormone content and hormonal balance. Our studies showed significantly lower concentration of ABA in etiolated plant material.

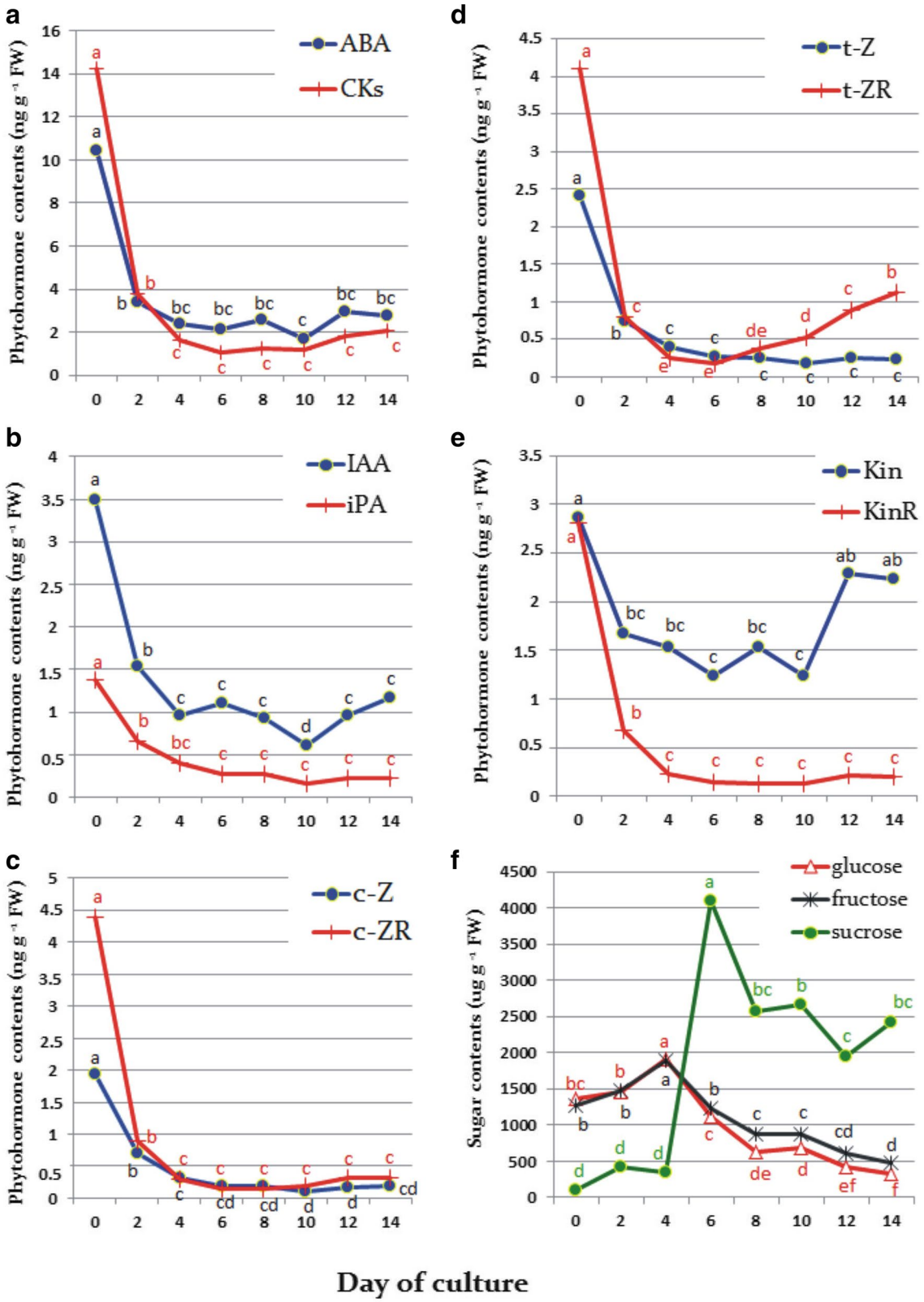

Fig. 2 Concentrations of phytohormones and soluble sugars during early somatic embryogenesis (14 days of culture in darkness). Data are the means of three replicates. Mean values marked with the *same letter* do not differ significantly at the 0.05 confidence level according to Kruskal–Wallis ANOVA and Median test. *ABA* abscisic acid, *CKs* the sum of c-Z, c-ZR, iPA, Kin, KinR, t-Z and t-ZR concentrations, *c-Z* cis zeatin, *c-ZR* cis zeatin riboside, *IAA* indole-3-acetic acid, *iPA* isopentenyladenosine, *Kin* kinetin, *KinR* kinetin riboside, *t-Z* trans zeatin, *t-ZR* trans zeatin riboside

Table 2 Ratios of different phytohormones in etiolated explants during 14-day-long culture of *C. delgadii*

Phytohormone ratios	Day of culture							
	0	2	4	6	8	10	12	14
IAA/CKs	**0.5 ± 0.06c**	**0.7 ± 0.01b**	**0.8 ± 0.02b**	**1.4 ± 0.16a**	**1.0 ± 0.16b**	**0.8 ± 0.12b**	**0.6 ± 0.04c**	**0.8 ± 0.01c**
IAA/t-Z	1.4 ± 0.50c	2.0 ± 0.09c	2.4 ± 0.22bc	4.2 ± 1.73ab	3.9 ± 0.43b	3.5 ± 1.31b	3.6 ± 0.44b	4.9 ± 0.74a
IAA/c-Z	1.8 ± 0.79d	2.1 ± 0.13d	3.1 ± 0.37c	6.17 ± 2.5a	5.5 ± 2.0b	5.7 ± 1.5ab	5.6 ± 1.5ab	6.1 ± 0.5a
IAA/t-ZR	0.8 ± 0.37c	2.0 ± 0.17bc	3.8 ± 0.75b	5.9 ± 1.74a	2.4 ± 0.28b	1.2 ± 0.21c	1.1 ± 0.48c	1.0 ± 0.1c
IAA/c-ZR	0.8 ± 0.37e	1.7 ± 0.1d	3.1 ± 0.38c	7.3 ± 2.22a	6.7 ± 1.13b	3.6 ± 1.81c	3.0 ± 0.4c	3.8 ± 0.49c
IAA/iPA	2.4 ± 0.39c	2.3 ± 0.17c	2.4 ± 0.05c	4.2 ± 1.66ab	3.5 ± 0.54b	3.9 ± 1.11b	4.2 ± 0.51ab	5.4 ± 0.69a
IAA/Kin	1.2 ± 0.17a	0.9 ± 0.14ab	0.6 ± 0.02b	0.9 ± 0.30ab	0.6 ± 0.15ab	0.6 ± 0.39ab	0.4 ± 0.03ab	0.5 ± 0.02ab
IAA/KinR	1.2 ± 0.38d	2.3 ± 0.19c	4.1 ± 0.60b	8.3 ± 3.91a	7.8 ± 2.40ab	5.5 ± 3.35b	4.5 ± 0.09b	6.1 ± 1.17ab
ABA/CKs	**0.5 ± 0.17b**	**0.6 ± 0.23ab**	**0.7 ± 0.22ab**	**0.9 ± 0.32a**	**0.9 ± 0.39a**	**0.7 ± 0.13ab**	**0.7 ± 0.38ab**	**0.6 ± 0.17ab**
ABA/t-Z	4.4 ± 1.32c	4.7 ± 0.96c	6.0 ± 0.86bc	7.7 ± 0.97b	10.7 ± 2.3a	9.4 ± 1.67ab	11.0 ± 0.71a	11.3 ± 0.15a
ABA/c-Z	5.4 ± 1.12e	5.0 ± 0.93e	7.7 ± 1.3d	11.2 ± 1.39c	15.9 ± 7.3bc	15.4 ± 1.14b	17.1 ± 3.66a	14.1 ± 0.77b
ABA/t-ZR	2.6 ± 0.52c	4.7 ± 1.6bc	9.5 ± 2.35a	11.1 ± 2.59a	6.7 ± 0.14b	3.2 ± 0.04c	3.4 ± 1.32c	2.5 ± 0.58c
ABA/c-ZR	2.4 ± 0.42e	4.1 ± 1.27d	7.8 ± 1.36c	13.8 ± 3.13b	19.01 ± 5.1a	9.53 ± 2.961c	9.4 ± 0.68c	8.8 ± 0.08c
ABA/iPA	8.4 ± 4.3c	5.4 ± 0.93d	5.9 ± 0.43d	7.7 ± 1.04c	9.8 ± 2.49b	10.6 ± 0.9ab	12.9 ± 0.81a	12.5 ± 0.13a
ABA/Kin	4.1 ± 2.17a	2.1 ± 0.18b	1.5 ± 0.12b	1.7 ± 0.34b	1.8 ± 0.60b	1.6 ± 0.66b	1.3 ± 0.03b	1.2 ± 0.11b
ABA/KinR	3.9 ± 1.31e	5.5 ± 1.84d	10.2 ± 2.01c	14.7 ± 0.84b	22.3 ± 9.07a	14.2 ± 6.03b	14.2 ± 0.58b	14.0 ± 0.77b
ABA/IAA	**2.7 ± 0.4a**	**2.2 ± 0.8ab**	**2.5 ± 0.2a**	**1.9 ± 0.5ab**	**2.8 ± 0.4a**	**2.8 ± 0.8a**	**3.0 ± 0.3a**	**2.3 ± 0.5ab**
Z-type/iPA	**9.9 ± 3.1a**	**4.8 ± 0.4c**	**3.2 ± 0.3c**	**2.9 ± 0.1c**	**3.6 ± 0.3c**	**6.3 ± 0.0b**	**7.5 ± 1.6a**	**8.6 ± 1.2a**

Data are the means (±SD) of three replicates. Mean values marked with the same letter do not differ significantly at the 0.05 confidence level according to Kruskal–Wallis ANOVA and Median test

Values marked with the same letter differ insignificantly at the 0.05 confidence level according to Kruskal–Wallis ANOVA and Median test

ABA abscisic acid, *CKs* the sum of c-Z, c-ZR, Kin, KinR, t-Z, t-ZR, and iPA concentrations, *c-Z* cis zeatin, *c-ZR* cis zeatin riboside, *IAA* indole-3-acetic acid, *iPA* isopentenyladenosine, *Kin* kinetin, *KinR* kinetin riboside, *t-Z* trans zeatin, *t-ZR* trans zeatin riboside, *Z-type* the sum of c-Z, c-ZR, t-Z, t-ZR concentrations

The results are in agreement with previously published data (Volmaro et al. 1998; Pacholczak et al. 2005). ABA is biosynthesised especially in chloroplasts and appears to be a cleavage product in the biosynthetic pathway of carotenoids (Seo and Koshiba 2002). In view of this, the reduction in ABA concentration in response to darkness seems to be related to changes in the structure and/or function of chloroplasts. Researchers also agree that the exposure of plants to darkness increases the concentration of endogenous IAA, even twice (Liu et al. 1996; Volmaro et al. 1998; Suzuki and Kerbauy 2006), and it is in agreement with the results obtained for *C. delgadii*. The negative effect of the light on IAA concentration is presumably because light induces IAA-peroxidase and IAA-oxidase activity, the key enzymes involved in the breaking down of IAA (Liu et al. 1996). Dark treatment also greatly stimulates cytokinin accumulation. In shoots of dark-grown *Catasetum fimbriatum*, the total cytokinin concentration was almost threefold greater than in those growing under photoperiodic conditions (Suzuki and Kerbauy 2006). In stipe explants of *C. delgadii*, this difference reached more than 9 in favour of the dark. Similarly, the concentrations of Z, ZR, iP, and iPA were significantly higher after the exposure to darkness than to continuous light (Macháčková et al. 1996). Our data confirmed this to be true also for other cytokinins, i.e. Kin and its riboside. Although the occurrence of Kin has been considered to be an artefact, its presence had already been found in plant material such as coconut chunks (Barciszewski et al. 1996) and water extracts (Ge et al. 2005), root nodules of Australian pine (Raman and Elumalai 1996) as well as triticale anthers (Żur et al. 2015). The importance of Kin in controlling different physiological processes in plants may be associated with its antioxidant and ABA-antagonistic properties (Barciszewski et al. 1996; Żur et al. 2015).

The key question of our research is how the content/ratio of phytohormones, established as a result of etiolation of the donor plantlets, promotes SE induction? The hormonal state of somatic cells determines the morphogenetic response of explants by genes classified as hormone-related transcription factors (Gliwicka et al. 2013). Some authors postulate that elevated level of ABA reduces/inhibits the explant ability to SE (Ivanova et al. 1994; Jiménez and Bangerth 2001a). According to this, our results showed the many times higher ABA/CKs and ABA/IAA ratios in non-embryogenic explants than in embryogenic explants. On the level of molecular studies, the negative relationship between the ABA content and the *LEC2* (*LEAFY*

COTYLEDON2) activity during SE induction was recently revealed by Wójcikowska and Gaj (2015). On the other hand, *LEC2* activation induces *YUC2* and *YUC4* (*YUCCA 2* and 4) genes involved in auxin biosynthesis (Stone et al. 2008). The connection between auxin and genes that are specific for early SE, such as *AGL15* (*AGAMOUS-Like15*) and *FUS3* (*FUSCA3*) has also been documented (Stone et al. 2008; Zheng et al. 2013). It is commonly known that an appropriate level of auxin is required for SE induction (Ayil-Gutiérrez et al. 2013; Gliwicka et al. 2013). Thus, the competence of *C. delgadii* explants to undergo SE may relate to low ABA level together with high content of IAA and cytokinins achieved by etiolation.

Explant excision resulting in altered endogenous hormone concentration

Our earlier research revealed that the tissue damage caused by aging and dying of the upper part of etiolated fronds can spontaneously induce direct or indirect SE in *C. delgadii* on PGR-free medium (Mikuła et al. 2015b). In plant tissue culture, wounding is one of the most important factors affecting the developmental programme of somatic cells (Iwase et al. 2011). Sometimes, this stress treatment may be enough to initiate SE (Smith and Krikorian 1989). Following a wound, the level of IAA in tobacco leaves declined 2- to threefold (Thornburg and Li 1991). Moreover, enhanced endogenous ABA, jasmonic acid, and ethylene production were detected (León et al. 2001). Wounding also enhanced the endogenous cytokinin activity (Crane and Ross 1986; Iwase et al. 2011). However, the hormonal response to excision seems to be completely different than to wounding. An excision releases explants from the influence of apical meristems that are the sources of hormone synthesis. Therefore, in excised stipes of *C. delgadii* about threefold reduction in the concentration of IAA, ABA and CKs was observed. The similar decline in IAA content was also noticed in excised cotton hypocotyl explants (Yang et al. 2012). Furthermore, it is worth mentioning that although a dramatic decline in the hormone concentration occurred, the balance between IAA/CKs, ABA/CKs and ABA/IAA ratios remained almost unchanged, and significantly greater concentration of CKs than IAA was still maintained in *C. delgadii* explants.

Summary of physiological events during early SE of *C. delgadii*

Analysis of the induction phase of SE from the viewpoint of physiological changes reveals three sub-phases (**I-III**) that can be observed in *C. delgadii* explants, and these are triggered by etiolation, explant excision, and a massive increase in endogenous sucrose concentration (Fig. 3).

Our results provide evidence that by etiolation of donor plantlets it is possible to achieve the hormonal balance which requires only a slight further modification to switch the developmental path of somatic cells. The etiolation reduces a high concentration of ABA, the main factor inhibiting somatic embryo production (Charrière et al. 1999; Jiménez and Bangerth 2001a), and increases the content of IAA and cytokinins. As a result of explant excision a dramatic decline in the concentration of studied hormones occurs, but the hormonal balance and sugar content remain almost unchanged. Among those phytohormones, the rapid decline in IAA concentration seems to be directly related to the sugar influx into the explant cells. It was shown that the low level of IAA leads to an increase in apoplastic pH and the activation of invertases (Mohr and Schopfer 1995). Seeing that, almost 12-fold increase in sucrose concentration due to the influx of glucose and fructose into the explant cells of *C. delgadii*, may represent an osmotic signal that is required for the expression of embryogenic competence (Kikuchi et al. 2006). We suggest that the short-term osmotic signal is a final switch to the massive genetic reprogramming towards embryogenesis. The studies also showed that the predominant class of hormones found in the stipe explants during early SE are cytokinins. In all likelihood, they favour this type of morphogenetic response, however, initiation of third sub-phase of induction phase seems to be supervised by auxin, as apparent from the highest IAA/CKs ratio on day 6 of culture.

In *C. delgadii*, the hormonal balance established at 6th day of culture (Fig. 3) promotes transition from the induction to the expression phase four days later. During next four days of culture many somatic embryos at the linear stage of development were present. This phase requires a considerable amount of metabolic energy and carbon skeletons (Gomes et al. 2014; Cangahuala-Inocente et al. 2009). Our results confirmed it by observation of intensive utilisation of soluble sugars. Third part of acquisition phase and an early stage of SE expression were also correlated with a greater concentration of cytokinins Z-type compared with iPA. This result resembles previous data indicating that the ratio of these two types of cytokinins is a good index of both the explant and callus embryogenic capacity (Centeno et al. 1997; Pintos et al. 2002). Among Z-type cytokinins, t-ZR appears to be particularly associated with frequent cell divisions leading to the production of linear stage somatic embryos in *C. delgadii*.

Conclusion

1. The capacity of stipe explants to form somatic embryos in *C. delgadii* is determined by the endogenous level and balance of hormones that are modulated by the

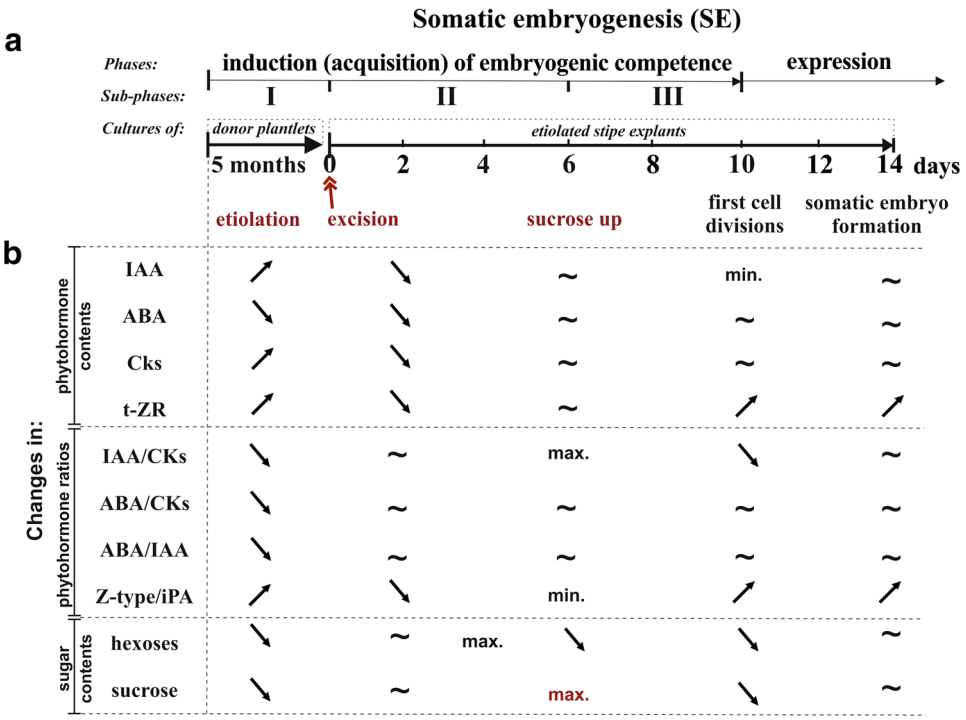

Fig. 3 Schematic summary of physiological events happened during early somatic embryogenesis in *C. delgadii*. **a** Time-line of embryogenic pathway development illustrating the two different phases, i.e. the induction (acquisition) of embryogenic competence and the expression of SE. The acquisition phase is divided into three sub-phases (I–III). There are also three different stimuli: etiolation, excision (exogenously applied), and a massive increase in endogenous sucrose concentration (sucrose *up*). They switch these sub-phases on and off. **b** Changes in endogenous hormone and sugar contents, and in hormone ratios, showing the influence of the stimuli. *ABA* abscisic acid, *CKs* the sum of c-Z, c-ZR, iPA, Kin, KinR, t-Z and t-ZR concentrations, *c-Z* cis zeatin, *c-ZR* cis zeatin riboside, *IAA* indole-3-acetic acid, *iPA* isopentenyladenosine, *Kin* kinetin, *KinR* kinetin riboside, *t-Z* trans zeatin, *t-ZR* trans zeatin riboside, *Z-type* the sum of t-Z, c-Z, t-ZR, c-ZR concentrations, *min./max.* minimum/maximum content, ↑/↓/~ increasing/decreasing/constant trend

conditions of light under which the donor plantlets have developed.

2. Drastic reduction in hormone contents caused by excision is sufficient to trigger embryogenic competence in epidermal cells of *C. delgadii* stipe explants.
3. Rapid increase in sucrose content can be considered as a switch to the SE expression phase.
4. *Trans*-zeatin riboside is clearly associated with SE expression phase of *C. delgadii* in which the frequent cell divisions happen and the somatic embryos at linear stage appear.

Acknowledgements The authors thank Professor Jan J. Rybczyński and Dr. Karolina Tomiczak (PAS Botanical Garden CBDC in Powsin, Warsaw, Poland) for their valuable advice and creative discussions during preparation of the manuscript. We would like to give special thanks to anonymous Reviewer 2 for valuable comments and suggestions. This research was supported by the Polish National Science Centre (NCN), no. 2011/03/B/NZ9/02472.

Author Contributions MG performed the experiments and prepared the samples for HPLC analysis. AK performed HPLC analyses of carbohydrates, PW carried out HPLC separation of phytohormones. AK and PW performed all chromatographic data analysis. MG and AM designed the experiments and wrote the manuscript. AM conceived the study. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Acta Physiologiae Plantarum (2018) 40:1
https://doi.org/10.1007/s11738-017-2577-4

ORIGINAL ARTICLE

Effect of TIBA, fluridone and salicylic acid on somatic embryogenesis and endogenous hormone and sugar contents in the tree fern *Cyathea delgadii* Sternb.

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Received: 12 June 2017 / Revised: 19 September 2017 / Accepted: 15 November 2017 / Published online: 22 November 2017

Abstract

Somatic embryogenesis (SE) in the tree fern *Cyathea delgadii* was first described in 2015 and since then has been used to exploration of this phenomenon in cryptogamic plants. To deepen the knowledge about the hormonal control of SE, stipe explants were cultured on media supplemented with hormone biosynthesis and transport inhibitors (HBTIs). In the presence of 30 µM 2,3,5-triiodobenzoic acid (TIBA), or 40 µM fluridone or 125 µM salicylic acid (SA), somatic embryo production was totally inhibited. The quantitative analysis of the changes in endogenous hormone and sugar contents was conducted every 2 days within 10-day-long initial culture. The results showed that the concentrations of endogenous indole-3-acetic acid (IAA), abscisic acid (ABA), cytokinins (CKs) and soluble sugars were strongly modified either by TIBA and fluridone. Under their influence, the contents of cytokinins such as c-Z, c-ZR, t-Z, t-ZR, KinR were reduced to barely detectable levels. Treatment with SA results in the changes in endogenous IAA and sugar contents. It also modifies the IAA/CKs ratio; however, excluding the first 2 days of culture, the concentrations of ABA and cytokinins were kept on the control level. All HBTIs significantly increased the kinetin (Kin) content. Our work sheds new light on the relationships between the biosynthetic inhibitors and phytohormones and sugars in the process of early SE. It can be helpful to study the role of hormones in acquisition of embryogenic competence.

Keywords Abscisic acid · Auxin · Cytokinins · Kinetin · Sucrose

Abbreviations

ABA	Abscisic acid
c/t-Z	*Cis/trans* zeatin
c/t-ZR	*Cis/trans*-zeatin riboside
CKs	Total concentration of cytokinins
Fluridone	1-Methyl-3-phenyl-5-[3-trifluoromethyl (phenyl)]-4-(1H)-pyridinone
FW	Fresh weight
HBTIs	Hormone biosynthesis and transport inhibitors
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
iPA	Isopentenyladenosine
Kin	Kinetin
KinR	Kinetin riboside
SA	Salicylic acid
SE	Somatic embryogenesis
TIBA	2,3,5-Triiodobenzoic acid

Communicated by M. Lambardi.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11738-017-2577-4) contains supplementary material, which is available to authorized users.

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Introduction

The most critical event in induction of somatic embryogenesis (SE) seems to be the establishment of a favourable hormonal balance within the initial explants cultured in vitro. Several external stimuli, such as plant growth regulators (Ayil-Gutiérrez et al. 2013), osmotic agents (Kikuchi

et al. 2006), nutritional components (Pěnčík et al. 2015), amongst others, have been recognised as essential factors in determining both the hormone biosynthesis and the developmental fate of explant cells. Therefore, many previous studies focused on the role of phytohormones on different aspects of SE (Jiménez 2005; Vondráková et al. 2016), but the endogenous hormonal regulation of SE induction (i.e., the phase in which the somatic cells undergo dedifferentiation and acquire an embryogenic competence) is still not well understood.

Hormone biosynthesis and transport inhibitors (HBTIs) are useful compounds for elucidating the effects of different phytohormones on SE (Jiménez 2005). By applying antiauxin or auxin polar transport inhibitors, many investigators have shown the necessity of this group of hormones for embryo maturation and its normal development (Vondráková et al. 2016). It has been also proposed that auxin may act as a trigger in the embryogenic program of plant somatic cells (LoSchiavo et al. 1989; Tokuji and Kuriyama 2003; Nic-Can and Loyla-Vargas 2016). Establishment of an auxin gradient was essential for initiating somatic embryos in *Eleutherococcus senticosus* Maxim. (Choi et al. 2001) and for inducing stem cell formation within the embryonic callus of *Arabidopsis* (Su and Zhang 2009). Conversely, it does not appear to be required for the very early stages of direct SE in *Daucus carota* L. (Tokuji and Kuriyama 2003). Like auxin, cytokinins are key regulators for various aspects of plant growth and development, including somatic embryo production (Jiménez 2005). Together with auxin, they regulate meristem formation in early embryogenesis. However, their importance during the acquisition of embryogenic potential is poorly understood. Tokuji and Kuriyama (2003) showed that cytokinins regulate the early stage of SE in carrot. Somleva et al. (1995) and Sáenz et al. (2010) have found an inverse relationship between cytokinins and the embryogenic response. Recent studies revealed a close link between auxin and ethylene in the mechanism that controls an acquisition of embryogenic competence on the level of specific genes (Nowak et al. 2015). Using salicylic acid (SA), that is considered as an inhibitor of ethylene biosynthesis, both the positive (Luo et al. 2001; Quiroz-Figueroa et al. 2001; Sakhanokho et al. 2009; Mulgund et al. 2012) and negative (Nissen 1994; Hosseini et al. 2011; Kępczyńska and Zielińska 2013) influence of the ethylene on somatic embryo production has been shown. In the case of abscisic acid (ABA), its role in SE initiation is less well known. So far, it has been shown that an accumulation of high levels of ABA inhibits the ability of the explant to undergo SE (Ivanova et al. 1994; Jiménez and Bangerth 2001; Grzyb et al. 2017). In conifers, the decline of ABA level in the presence of the ABA synthesis inhibitor fluridone enhances the transition of proembryogenic masses to early somatic embryos (Farias-Soares et al. 2014). However, some ABA seems to be necessary for the initiation of both direct and indirect SE (Rajasekaran et al. 1987; Kikuchi et al. 2006; Su et al. 2013). Although the biosynthetic inhibitors are often used in study of SE, there are only a few reports describing how the contents of different endogenous hormones are altered by treatment with those substances (Rajasekaran et al. 1987; Nissen 1994; Kikuchi et al. 2006; Ruduś et al. 2009; Farias-Soares et al. 2014).

It is worth noting that the regulatory role of ABA is achieved, in part, by crosstalk with other hormones (Belin et al. 2009), and with sucrose signalling pathways involved in starch biosynthesis. On the other hand, sugars modulate many vital processes, including embryogenesis, via crosstalk with phytohormones (Gibson 2004). For example, the availability of sucrose and glucose helps regulate the transition from growth by cell division to growth by cell expansion and reserve accumulation in developing embryos (Gibson 2004). Moreover, the role of *LEAFY COTYLEDON1* (*LEC1*) gene, that controls embryogenic competence, requires both the auxin and sucrose to promote cell division and embryonic differentiation (Casson and Lindsey 2006). Various exogenous sugars have been used to enhance SE efficiency, indicating that carbon sources play an important role in this process (Yaseen et al. 2013). Despite the importance of sucrose metabolism in maintaining the balance between hexose signals and metabolic paths (Koch 2004), there is only one example of the relationship between the content of phytohormones and endogenous sugars during the SE induction currently displayed (Grzyb et al. 2017).

The tree fern *Cyathea delgadii* Sternb. is the first cryptogamic plant to be used as a model system for studying SE (Mikuła et al. 2015b; Domżalska et al. 2017; Grzyb et al. 2017). It is also one of the few plant species in which direct SE can be induced on plant growth regulator-free medium (Mikuła et al. 2015a). The hormonal balance that allows induction of embryogenic potential in *C. delgadii* is achieved by long-term etiolation of donor plantlets (Mikuła et al. 2015a). It reduces a high concentration of phytohormone ABA—the main factor inhibiting the explant ability to SE (Grzyb et al. 2017). In response to an explant excision, a dramatic reduction in the content of several cytokinins, indole-3-acetic acid (IAA) and ABA happen. It initiates the whole cascade of events leading to the acquisition of embryogenic competence. Among them the sudden increase in the concentration of soluble sucrose is considered to be the switch to phase in which certain epidermal cells regain their potential to division and to develop into somatic embryos. In this way, the explants of juvenile etiolated stipes of *C. delgadii* undergo restructuring to generate embryogenic cells during 10 days of initial culture (Grzyb et al. 2017). These studies have shown that the balance between the endogenous IAA and cytokinin content is particularly related to the induction phase of SE. In the present work, the hormone biosynthesis and transport inhibitors, such as TIBA, fluridone and SA,

were used to examine their impact on the somatic embryo production in *C. delgadii*. The effect of these treatments on endogenous contents of IAA, cytokinins, ABA and sugars in stipe explants is presented.

Materials and methods

Plant material and tissue culture

The somatic embryo-derived sporophytes of *C. delgadii* were cultured on agar medium containing half-strength Murashige and Skoog's (1962) macro- and micro-nutrients and a full complement of vitamins (1/2MS), supplemented with 2% (w/v) sucrose; 0.7% plant agar (Duchefa Biochemie); pH 5.8. The cultures were maintained in constant darkness, in a climatic chamber at +24 ± 1 °C. Five-month-old etiolated plantlets that had developed 4–5 leaves, were used as a source of explants (Mikuła et al. 2015a, b). Stipe explants measuring 2.5 mm in length were excised from the first frond of etiolated sporophytes and cultured on 1/2MS medium with 1% (w/v) sucrose (control), and supplemented with TIBA (4, 8, 10, 12, 16, 20 and 30 µM) or fluridone (1, 10, 20, 30 and 40 µM) or SA (1, 5, 10, 25, 50, 75, 100 and 125 µM), in darkness. TIBA was dissolved in 0.1% (v/v) dimethyl sulfoxide (DMSO). Medium supplemented with DMSO, that was used as additional control, did not inhibit the SE.

The efficiency of SE was expressed as the percentage response of explants, calculated as follows: (number of explants that formed somatic embryos/total number of explants) × 100; and as the number of somatic embryos per responding explant. Somatic embryos were counted under a stereo microscope (Olympus SZH, Japan) following 2 months of culture.

Sample preparation for HPLC analysis

The samples were collected every 2 days within 10-day-long culture. Immediately after collection, the stipe explants (about 80 explants per sample; 35 mg of FW) were frozen in liquid nitrogen, lyophilised and homogenised in an ice-cold mixture of methanol, water, and formic acid (15/4/1) according to Dobrev and Kamínek (2002). Internal isotopic standard mixture consisting of deuterated IAA and Kin labelled with nitrogen ^{15}N was added to each sample. The prepared extract was fractionated using solid phase extraction (SPE) columns Oasis MCX (Waters). According to HPLC method of Štefančič et al. (2007), Żur et al. (2015), and Hura et al. (2016), three fractions were collected: "acidic" for IAA and ABA analyses, "basic" for cytokinins analyses, and "flow through" for carbohydrates analyses, as provided by Grzyb et al. (2017). All chemicals for sample preparation and HPLC were bought in the Sigma-Aldrich Sp. z.o. (Poznan, Poland), HPLC standards (normal and stable isotope labelled) were bought in the Olchemim (Olomouc, Czech Republic).

Quantification of IAA and ABA

The fraction containing IAA and ABA was eluted from SPE column according to Żur et al. (2015). The samples were analysed using a Supelco Ascentis RP-Amide column (7.5 cm × 2.1 mm, 2.7 µm) as has been previously described (Grzyb et al. 2017). The HPLC apparatus used was Agilent Technologies 1290 Infinity equipped with Agilent Technologies 6460 Triple Quad LC/MS with Jet Stream. Two most abundant ions were monitored (MRM—multiple reaction monitoring mode) and they were: IAA—176.1 primary, 130.3, 77.2 secondary; D-IAA (deuterated IAA used as internal standard)—181.1 primary, 134.7, 81.4 secondary; ABA—265.2 primary, 229.1, 247.1 secondary. Agilent Technologies Mass Hunter 5 software was used for apparatus control, data collection, and processing.

Quantification of cytokinins

Cytokinins such as c-Z, c-ZR, iPA,t-Z, t-ZR, Kin and KinR were separated from the samples. Their sum is defined as the total concentration of cytokinins (CKs). In order to assess hormonal changes introduced by HBTIs, the phytohormone balance was expressed by the ratios of IAA to total amount of CKs, and ABA to CKs or IAA.

Fractions of cytokinins were flushed out from the SPE column after collecting IAA and ABA, collected using procedure described earlier (Grzyb et al. 2017) and analysed using the same chromatographic system and HPLC column as described above. The monitored ions were as follows: c/tZ—220.2 primary, 136.3, 202.3, secondary; c/t ZR—352.4 primary, 220.3, 136.3, secondary; Kin—216.2 primary, 188.3, 148.3 secondary; KinR—348.3 primary, 148.3, 216.3 secondary; iPA—204.2 primary, 136.3, 148.3 secondary; Kin-^{15}N (heavy nitrogen labelled Kin used as internal standard)—220.1 primary, 192.3, 152.3 secondary.

Quantification of soluble sugars

The "flow through" fraction collected from the SPE column was lyophilised and reconstituted according to the procedure described by Janeczko et al. (2010). Samples were analysed using Agilent Technologies 1200 HPLC equipped with an amperometric detector ESA Coulochem II Analytical Cell 5040 with gold electrode. A Hamilton RCX-10 250 × 4.1 mm (Hamilton, Reno, NV, USA) HPLC column was used. The mobile phase, flow rate of HPLC column and the detector settings have been applied according to Grzyb

et al. (2017). Agilent Technologies ChemStation B04 software was used for apparatus control, data collection and processing.

Statistical analysis

Data for the effect of HBTIs on somatic embryo production are presented as mean ± SD for three biological replications each consisting of 48 explants, and these were tested by means of one-way ANOVA analysis of variance and Fisher's least significant difference (LSD) procedure using Statgraphics Plus software. Significance was set at a P value of less than 0.05. Data for endogenous hormone and sugar contents are presented as mean ± SD for three replicates (35 mg FW, i.e., about 80 explants, was considered as one replicate). Differences in the concentration of hormones and sugars between the control and HBTIs treatments were analysed using the Student's t test for each day separately. Only a return of $P < 0.05$ was designated as being statistically significant. Statistical analysis was performed for each type of HBTIs separately.

Results

Effect of TIBA, fluridone and SA on the somatic embryo production in *C. delgadii*

After 2 months of culture on control medium, 78.5% of explants were able to produce somatic embryo-derived sporophytes showing an efficiency of almost 25 embryos per responding explant (Figs. 1a, 2a). Those sporophytes developed the first leaf, shoot apex and primordium of the second leaf, as well as root primordium (Fig. 1b). Although

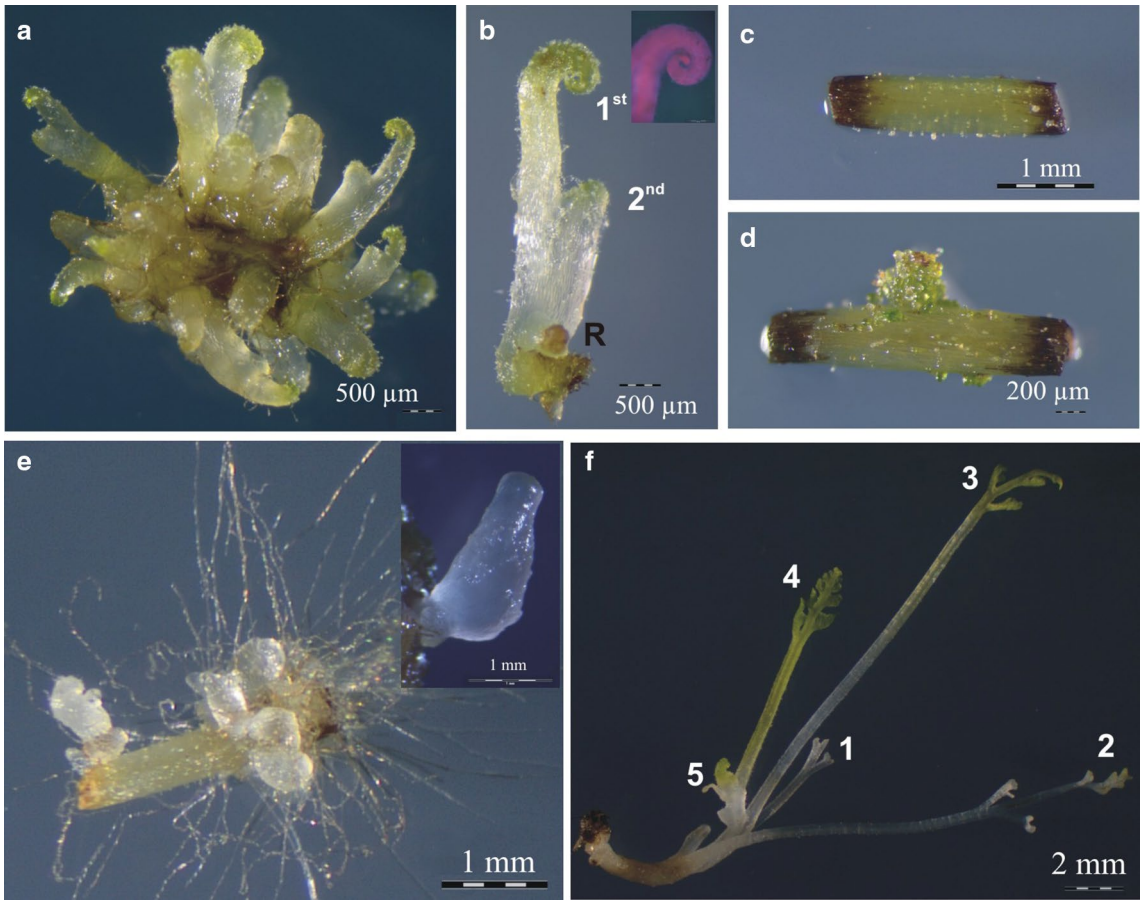

Fig. 1 Somatic embryogenesis (SE) of *Cyathea delgadii* and its disturbances. **a**–**e** Cultures kept in the dark for 2 months. **a** Numerous somatic embryo-derived sporophytes obtained on ½ MS medium. **b** Details of juvenile sporophyte development; greenish crosier of the first frond and root on the base of sporophyte are visible. The inset shows red autofluorescence of chlorophyll in crosier cells induced by blue-violet light (blue-violet light; BV filter 400–440 nm). **c** Stipe explant after 2 months of culture in the presence of 30 µM TIBA without any cell divisions and **d** with gametophyte-like structures. **e** Whitish somatic embryos formed in the presence of 10 µM fluridone (details on inset). **f** Whitish frond of sporophyte (1 and 2) developed in darkness on the medium supplemented with fluridone. Green primordium (5), frond (4) and leaf blade (3) formed after transferred of sporophyte to fluridone-free medium and kept under photoperiodic conditions (5-month-old plantlet). *R* root, *1st* first leaf, *2nd* second leaf

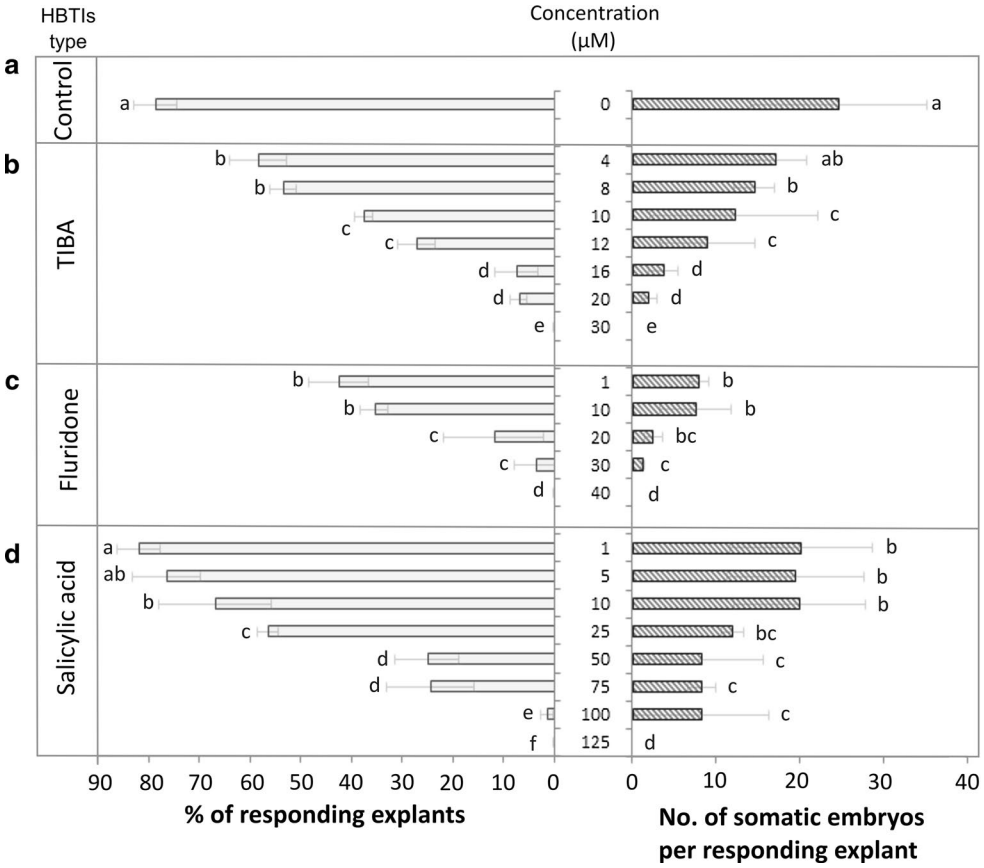

Fig. 2 Efficiency of somatic embryogenesis in *C. delgadii* after 2 months of culture on control medium (1/2MS medium supplemented with 1% sucrose) (**a**) or in the presence of TIBA (**b**), fluridone (**c**) or SA (**d**); in darkness. *HBTIs* hormone biosynthesis and transport inhibitors, *TIBA* 2,3,5-triiodobenzoic acid. Values marked with the same letter do not differ significantly at the 0.05 level according to Fisher's least significant difference (LSD's) test. Data represent mean ± standard deviation of three independent experiments, each consisting of 48 explants

the culture was kept in the dark, the first leaf of the juvenile sporophyte was clearly greenish, but the blades of the fronds were characteristically coiled in the crosier at all times (Fig. 1a, b).

The capacity to produce somatic embryos gradually declined with increasing concentrations of TIBA and fluridone (Fig. 2b, c). The percentage of responding explants and the number of somatic embryos were about 2-fold less in explants treated with 10 μM TIBA or fluridone than in controls. The embryogenic capacity was totally lost in the presence of 30 μM TIBA or 40 μM fluridone. There were no specific cell divisions for somatic embryo formation observed (Fig. 1c, d). The somatic embryos that were formed on the explants treated with a smaller dose of fluridone were unable to synthesise functional chlorophyll as indicated by their pallid appearance (Fig. 1e). It led to the formation of sporophytes with completely white fronds. The sporophytes, after being transferred to fluridone-free medium and exposed to light, produced normally developed green leaves with expanded leaf blades (Fig. 1f).

When SA was used at concentrations of 1–5 μM, there were no significant differences in the percentage of responding explants observed, and only a slight decrease in the number of somatic embryos was achieved (Fig. 2d). Concentrations of SA between 50 and 100 μM resulted in a dramatic reduction in the embryogenic capacity of *C. delgadii* stipe explants. The medium supplemented with 125 μM SA completely inhibited SE. The gametophyte-like structures were formed sporadically at the end of first month of SA-treated culture (Fig. 1d).

Effect of TIBA, fluridone and SA on the content of endogenous IAA, ABA, cytokinins and sugars

In order to study the involvement of endogenous IAA, ABA, cytokinins and sugars in SE induction of *C. delgadii*, TIBA, fluridone and SA were used at concentrations that totally inhibited somatic embryo production, i.e., 30, 40 and 125 μM, respectively. When these HBTIs were added to the induction medium, the content of all phytohormones studied diminished rapidly in excised explants during 2 days of culture (Fig. 3a–c; Supplementary Fig. 1). In the case of explants cultured on control medium, minimal levels of these hormones were achieved 2 days later.

The use of TIBA contributed to about a 2-fold reduction in the concentration of IAA and CKs (Fig. 3a, c). A total reduction in the content of c-Z, c-ZR, t-Z, t-ZR, KinR was also observed (Supplementary Fig. 1). Moreover, almost a 4-fold increase in ABA content between days 6 and 8 of culture was found (Fig. 3b). TIBA also reduced

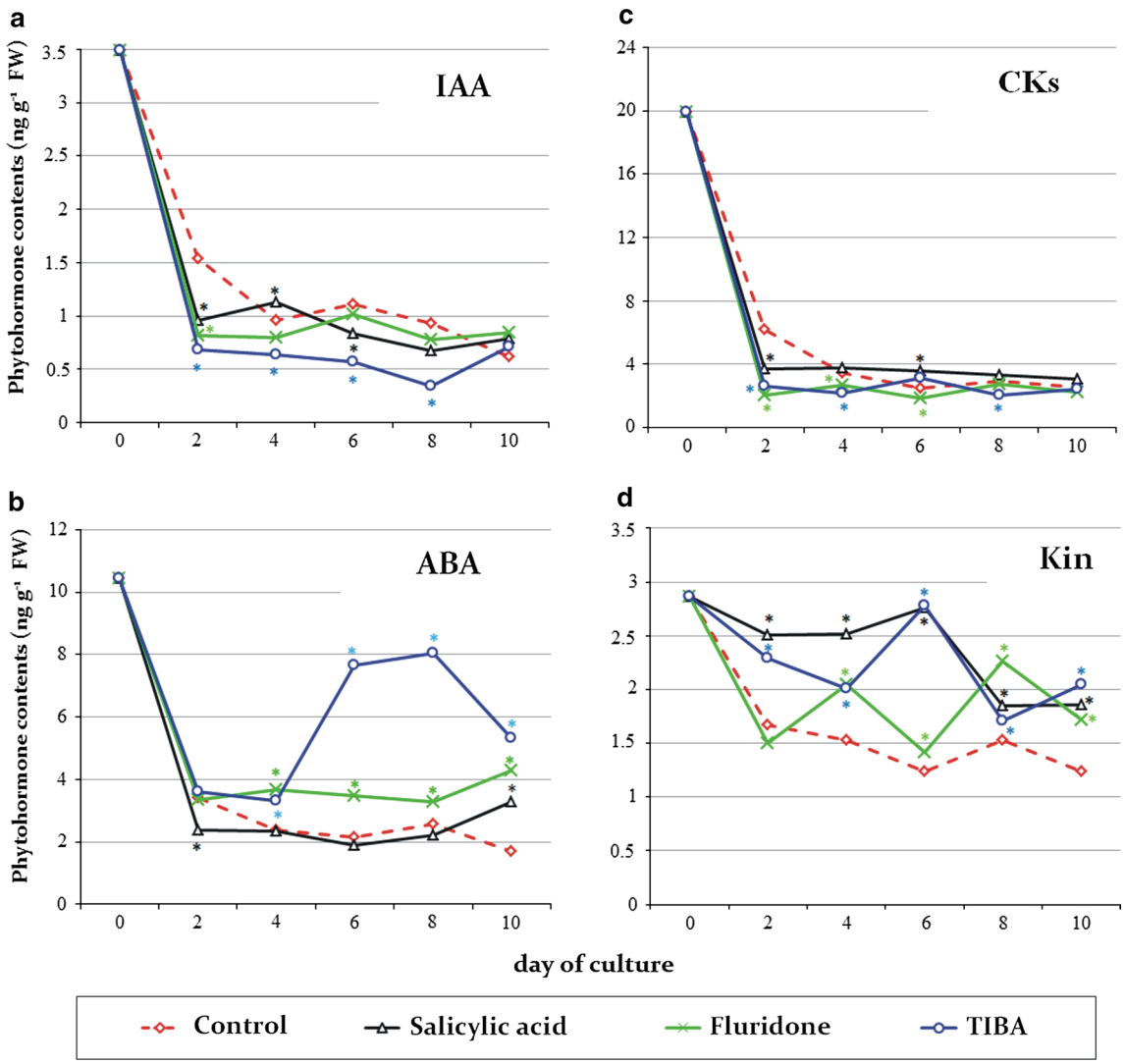

Fig. 3 The effect of SA, fluridone and TIBA on endogenous **a** IAA, **b** ABA, **c** CKs and **d** Kin contents in the stipe explants of *C. delgadii* during 10 days of culture. *ABA* abscisic acid, *CKs* the sum of t-Z, c-Z, t-ZR, c-ZR, Kin, KinR and iPA contents, *IAA* indole-3-acetic acid, *Kin* kinetin, *TIBA* 2,3,5-triiodobenzoic acid. Values marked with asterisk differ significantly from the control at the 0.05 level according to Student's *t* test. Data represent mean ± standard deviation of three independent experiments, each consisting of 48 explants

the IAA/CKs ratio and showed an increasing trend in the ABA/CKs and ABA/IAA ratios peaking at day 8 of culture (Fig. 4a–c). Another characteristic feature of the TIBA treatment was complete consumption of sugars on day 2 (Fig. 4d–f). The sugar concentrations increased gradually over the next 6 days of culture.

When fluridone was added to the induction medium, the ABA and CKs contents showed a 2-fold increase and a 2-fold reduction, respectively, compared to control explants (Fig. 3b, c). These changes resulted in a 1- to 3-fold increase in the value of all hormone ratios (Fig. 4a–c). The sucrose concentration showed an increasing trend in parallel with a reduction in glucose and fructose content (Fig. 4d–f). At 4 days after commencing culture, the content of all sugars gradually diminished.

Application of SA practically did not affect the concentrations of ABA and CKs after day 4 of culture (Fig. 3b, c). Thus, the ABA/CKs and ABA/IAA ratios were similar to those observed in control explants throughout the duration of culture (Fig. 4b, c). The most important change noticed was 2.5-fold decrease in the value of IAA/CKs ratio starting on day 4 of culture (Fig. 4a). Significant changes in sugar content were also observed (Fig. 4d–f).

Regardless of the type of HBTIs added to the medium (excluding an effect of fluridone at day 2), the concentration of Kin was much higher than in control plant material throughout the entire length of the culture period (Fig. 3d).

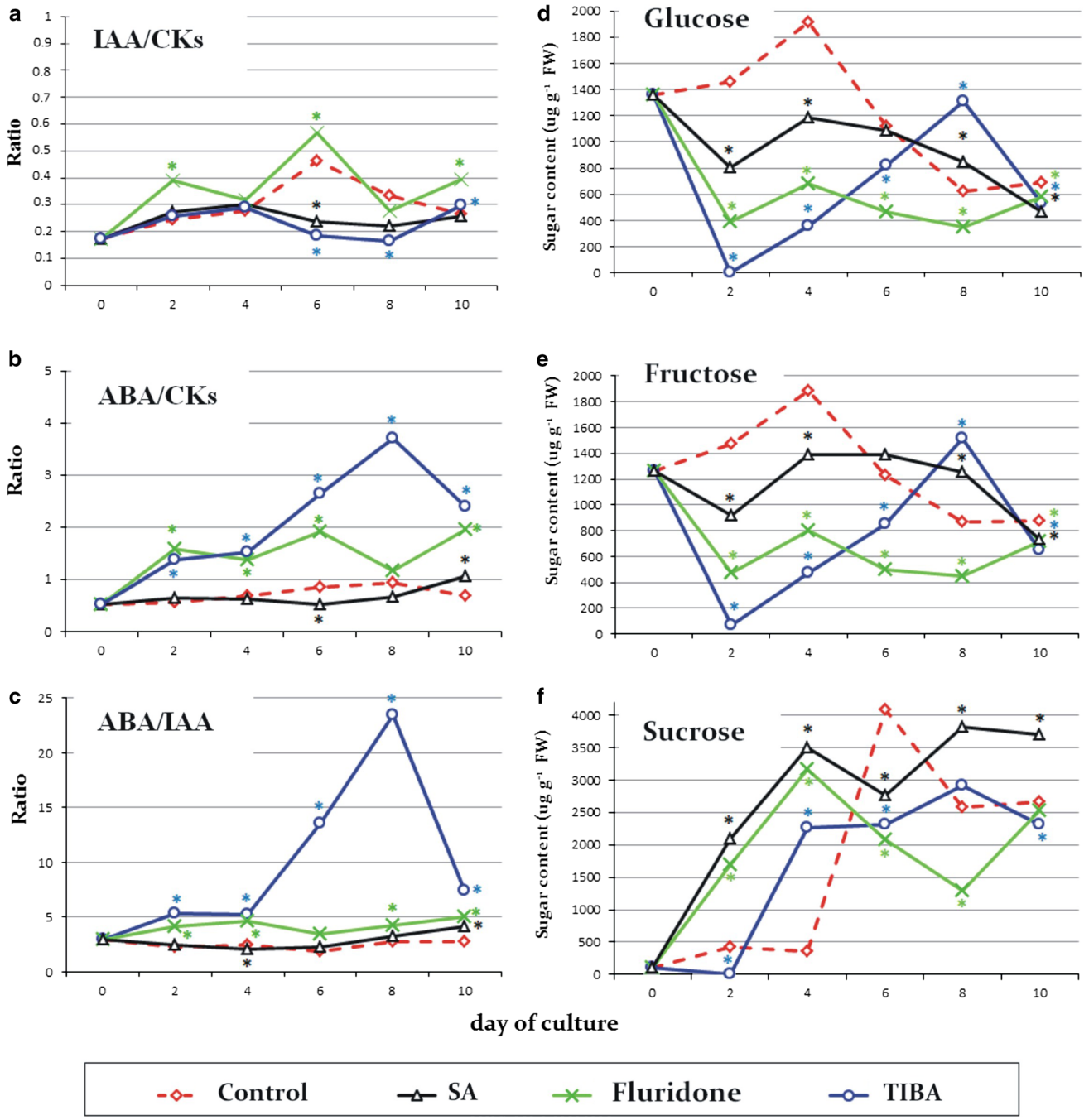

Fig. 4 Ratios between various phytohormones (**a**–**c**) and concentrations of soluble sugars (**d**–**f**) during 10 days of initial culture on control medium (1/2MS medium supplemented with 1% sucrose) or in the presence of SA, fluridone or TIBA. *ABA* abscisic acid, *CKs* the sum of t-Z, c-Z, t-ZR, c-ZR, Kin, KinR and iPA contents, *IAA* indole-3-acetic acid, *SA* salicylic acid, *TIBA* 2,3,5-triiodobenzoic acid. Values marked with asterisk differ significantly from the control at the 0.05 level according to Student's *t* test. Data represent mean ± standard deviation of three independent experiments, each consisting of 48 explants

Discussion

Modifications of the culture conditions through the inclusion of biosynthetic inhibitors affecting the polar transport of auxin and different hormone synthesis have been extensively used to investigate their role during somatic embryo production and development in spermatophytes. Here, HBTIs were used for the first time as a tool to investigate hormonal control of SE in ferns.

Effect of HBTIs on somatic embryo production

The auxin polar transport inhibitor TIBA competes with the auxin binding site on the efflux carriers. Its effect on SE depends on the genotype (Laublin et al. 1991) and explant type (Choi et al. 1997), as well as the dosage. TIBA at a concentration of 0.5 µM greatly increased the number of somatic embryos during direct SE of *Oncidium* (Chen and Chang 2004). At a concentration of 4–40 µM, TIBA was found to be inhibitory for the SE of *Eleutherococcus senticosus, Panax ginseng* C.A.Meyer, *Picea abies* (L.) H. Karst, and *Arachis hypogaea* L. (Laublin et al. 1991; Choi et al. 1997, 2001). In *C. delgadii*, a dramatic reduction in embryo-forming capacity was correlated with an increase in TIBA concentration. In the presence of 30 µM TIBA, somatic embryo production was completely inhibited.

Application of fluridone revealed that ABA is needed not only to promote maturation and normal development of somatic embryos (Rajasekaran et al. 1982; Nickle and Yeung 1994; Senger et al. 2001; Su et al. 2013), but also to the acquisition of embryogenic competence. Rajasekaran et al. (1987) and Su et al. (2013) demonstrated the inhibitory effect of fluridone at concentrations ranging from 0.1 to 3 µM on SE of *Pennisetum purpureum* Schum and *Arabidopsis.* In carrot stress-inducible SE, embryo formation was inhibited by 100 µM fluridone (Kikuchi et al. 2006). Our study showed that 40 µM fluridone is sufficient for total inhibition of SE in *C. delgadii*. It is worth emphasising that the inhibitory effect of fluridone can be nullified by the simultaneous application of fluridone and ABA, confirming the important role of this hormone in SE induction (Kikuchi et al. 2006). A significant problem with fluridone application is its inhibitory effect on carotenoid synthesis (Popova 1995). Lack of carotenoids results in a block in membrane formation and the concurrent blanching of plantlets. Here, our results indicate that this negative effect is irreversible. The newly formed leaves of blanched plantlets of *C. delgadii* transferred to fluridone-free medium and subjected to light became green and fully functional.

An effect of SA on SE induction appears to be strongly species- or genotype-dependent. In *Coffea arabica* suspension culture, picomolar concentrations of SA resulted in a 2-fold increase in cellular growth and SE efficiency, but 1 µM inhibited these processes (Quiroz-Figueroa et al. 2001). Incorporation of 7.2 µM SA in the induction medium increased the percentage of SE in ten genotypes of *Pinus roxburghii* (Chir pine), but concentrations between 14.5 and 36.3 µM had a toxic effect and resulted in the browning of explants (Malabadi et al. 2008). It was also shown that the inclusion of SA in the induction medium at concentrations of 40, 75–100, 150 and 500 µM enhanced SE in callus cultures of *Coffea canephora* P ex Fr., *Hedychium bousigonianum* Pierre ex Gagnep., *Astragalus adsurgens* Pall., and the formation of embryogenic callus in *Avena nuda* L., respectively (Luo et al. 2001; Hao et al. 2006; Kumar et al. 2007; Sakhanokho et al. 2009). In contrast to these findings, our results showed that the number of somatic embryos of *C. delgadii* diminished with increasing concentrations of SA. When SA was used at a concentration of 125 µM, SE was completely inhibited. Our findings are consistent with those obtained for *Daucus carota*, where 100 µM SA almost totally suppressed somatic embryo production (Hosseini et al. 2009).

The impact of biosynthetic inhibitors on the endogenous hormone and sugar contents

How the contents of different phytohormones are disturbed under the influence of inhibitor treatment is still an open problem. Following application of biosynthetic inhibitors in concentrations that stopped an expression of embryogenic totipotency in stipe explants of *C. delgadii*, it was possible to demonstrate the changes in content of endogenous IAA, ABA and cytokinins during first 10 days of initial culture. Selected phytohormones are well known as the key regulators of plant cell division and differentiation (Nic-Can and Loyola-Vargas 2016). Their signals may also affect sucrose metabolism (Lee and Huang 2013).

The earlier works showed the inhibitory effect of TIBA on the somatic embryo development by blocking the polar auxin transport (Schiavone and Cooke 1987; Liu et al. 1993). Some investigators have also suggested the necessity of polar auxin transport for initiation of embryo formation (Choi et al. 1997, 2001; Su and Zhang 2009). On the contrary, studies by Tokuji and Kuriyama (2003) showed that it is not needed in the induction of direct SE in carrot. Ramarosandratana and Van Staden (2004) hypothesised that the reduction of embryonal-suspensor mass initiation in *Picea abies* L. Karst. following application of TIBA resulted from an increase of internal auxin concentration rather than disruption of auxin gradient. However, our study clearly showed that exogenously applied TIBA at a concentration of 30 µM contributes to about a 2-fold reduction in the IAA content compared to a non-treated control. Furthermore, there was even a 4-fold increase in the level of ABA observed between days 6 and 8 of culture. TIBA also strongly modified the content of cytokinins and the ratios between all phytohormones studied here. To the best of our knowledge, this is the first report describing the relationship between TIBA treatment and endogenous hormone contents. We conclude that loss of ability to SE by explant treated with TIBA may be a direct result of the ABA accumulation in response to a stress.

When an early SE of *C. delgadii* was examined by the use of fluridone, we can clearly see that ABA interferes with auxin metabolism. In stipe explants treated with fluridone, the concentrations of endogenous IAA and ABA

are maintained at slightly lower, and at an almost 2-fold greater concentration, respectively, when compared with control explants. In young leaf explants of *Pennisetum purpureum* Schum. that were cultured in the presence of fluridone at concentration higher than 5 mg/l for 5 days, both the IAA and ABA levels decreased (Rajasekaran et al. 1987). These fluctuations are consistent with the trend presented for proembryogenic tissue of *Araucaria angustifolia* (Bertol.) (Farias-Soares et al. 2014) and for 14-day-old explants of cotyledonary somatic embryo of *Medicago sativa* L. (Ruduś et al. 2009). The negative effect of fluridone on the content of endogenous ABA was also shown in carrot explants (Kikuchi et al. 2006). It is difficult to explain why the *C. delgadii* explants treated with fluridone contain 2-fold more endogenous ABA than control explants. Our analysis showed that fluridone, like TIBA, not only disrupts the contents of IAA and ABA, but also reduces the contents of some endogenous cytokinins (such as c-Z, c-ZR, t-Z, t-ZR, KinR) to barely detectable levels.

SA is a stress-related phytohormone that functions as an important signalling molecule involved in the embryogenic response. Its activity induces an increase in the endogenous concentration of hydrogen peroxide (H_2O_2), and it has been proposed that it signals the initiation of SE (Luo et al. 2001). However, prolonging SA treatment can generate overproduction of H_2O_2 (Rao et al. 1997), and consequently inhibit SE. The inhibitory effect of SA on somatic embryo production in *C. delgadii* may also be related to the inhibition of the ethylene synthesis, as it was suggested by other authors (Romani et al. 1989; Quiroz-Figueroa et al. 2001; Hosseini et al. 2011; Mulgund et al. 2012). By using SA we revealed significant decrease in the content of endogenous IAA that was observed almost throughout the culture period. It is worth to note that the concentrations of ABA and cytokinins were lower than in control explants only during first 2 days of culture; however, the values of the hormonal ratios remained unchanged.

Our results provide evidence that by HBTIs treatment a significant increase in endogenous Kin content happens. Surprisingly, the high levels of Kin were reached under the influence of SA during first 6 days of culture. It may be the result of cellular changes involving DNA degradation or intensive oxidative metabolism (Barciszewski et al. 2007). It was shown that Kin is synthesised via the production of furfural, an oxidative damage product of DNA deoxyribose, and it is quenched by the adenine base (Barciszewski et al. 2000). Some data indicate its strong antioxidant properties (Olsen et al. 1999; Barciszewski et al. 2000; Żur et al. 2015).

In view of the interactions between hormone and sugar response pathways (Gibson 2004), we showed that treatment with HBTIs affects glucose, fructose and sucrose contents in stipe explants of *C. delgadii.* All HBTIs investigated caused significant changes to endogenous sugar profiles, the most important being the absence of a short-term increase in sucrose concentration at day 6 of culture. This rapid increase in sucrose content is considered as a switch to the SE expression phase in *C. delgadii* (Grzyb et al. 2017).

Conclusions

In the present investigation we showed that TIBA, fluridone and SA at concentrations 30 µM, 40 µM and 125 µM, respectively, totally suppress somatic embryo production in *C. delgadii*. Both TIBA and fluridone strongly influence the levels of endogenous IAA, ABA and cytokinins as well as the hormone ratios. Under their influence, the contents of c-Z, c-ZR, t-Z, t-ZR, KinR were reduced to barely detectable levels. Treatment with SA results in the changes in IAA content and the IAA/CKs ratio that are particularly related to the SE induction. The imbalances in phytohormone level are responsible for the modification of sugar contents, including sucrose—the main factor triggering embryogenesis in *C. delgadii*.

Our work sheds new light on the relationship between the treatment with biosynthetic inhibitors and the changes in phytohormone and sugar levels. It is worth to emphasise that this is the first study on influence of HBTIs on SE in cryptogams. It can be helpful to study the role of hormones in acquisition of totipotency.

Author contribution statement MG designed and performed the experiments. AK carried out HPLC separation. MG and AM wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements The authors thank dr. Piotr Waligórski (The Franciszek Górski Institute of Plant Physiology, PAS, Cracow, Poland) for help with HPLC analysis and Professor Jan J. Rybczyński (PAS Botanical Garden CBDC in Powsin, Warsaw, Poland) for valuable advice and creative discussions during preparation of manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Funding Polish National Center for Science (2011/03/B/NZ9/02472).

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Subject areas:
(1) growth and development
(8) cell–cell interaction

Number of figures: black and white – 0; color 6
Number of tables – 1

Symplasmic Isolation Contributes to Somatic Embryo Induction and Development in the Tree Fern *Cyathea delgadii* Sternb.

Symplasmic Isolation During Fern Somatic Embryogenesis

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Abstract

In this report, we describe studies on symplasmic communication and cellular rearrangement during direct somatic embryogenesis (SE) in the tree fern *Cyathea delgadii*. We analyzed changes in the symplasmic transport of low-molecular weight fluorochromes, such as 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS) and fluorescein (delivered to cells as fluorescein diacetate – FDA), within stipe explants and somatic embryos originating from single epidermal cells and developing during 16 days long culture. Induction of SE is preceded by a restriction in fluorochrome distribution between certain explant cells. Microscopic analysis showed a series of cellular changes like decrease of vacuole size, increase of vacuoles numbers, increased density of cytoplasm and deposition of electron-dense material in cell walls which may be related with embryogenic transition. In somatic embryo, the limited symplasmic communication between its cells was observed first in linear tri-cellular embryo. Further development of the fern embryo was associated with the formation of symplasmic domains corresponding to the four segments of the plant body. Using symplasmic tracers, we provided evidence that the changes in plasmodesmata permeability are corelated with somatic-to-embryogenic transition and somatic embryo development.

Keywords:

Cell wall, fluorochromes, somatic embryogenesis, ultrastructure

Introduction

Somatic embryogenesis (SE) is a process facilitating development of structures resembling the zygotic embryos from somatic cells of a plant body, which undergo dedifferentiation events and an orderly arrangement of characteristic developmental stages. This phenomenon has been extensively studied at the levels of genes, proteins, metabolites and morphology, including analyses of relationships between the cell wall, plasma membrane and cytoskeleton (Fehér 2015). Elucidation of mechanisms leading to the formation of somatic embryos is difficult due to the different pathways of SE initiation, and the strikingly different and highly sophisticated conditions that can trigger this process (Radoeva and Weijers 2014; Fehér 2015). A unique experimental system of SE in the tree fern *Cyathea delgadii*, described for the first time in 2015 (Mikuła et al. 2015b), can be a helpful tool to understand various events that occur during the somatic-to-embryogenic transition (Mikuła et al. 2018). *Cyathea delgadii* belongs to a small group of plants in which the explant cells can be reprogrammed *in vitro* to form somatic embryos in the absence of exogenous growth regulators (Mikuła et al. 2015a). The pathway of somatic embryo initiation and development in *C. delgadii* explants depends on such artless factors as length and diameter of the explant-donor frond, type of explant, as well as physical and chemical factors of media and culture conditions (Mikuła et al. 2015a; Grzyb and Mikuła, 2019). One of the basic triggers inducing SE are the light conditions for donor plantlet growth (Mikuła et al. 2015a) which regulate the endogenous hormone contents and hormonal balance (Grzyb et al. 2017). However, the most interesting feature of this model seems to be the epidermal and unicellular origin of somatic embryos (Mikuła et al. 2015b). Studies on *C. delgadii* showed that the first cellular divisions leading to the formation of somatic embryo are different from those occurring during zygotic embryogenesis. The early stage of SE is characterized by several cell divisions perpendicular to the apical-basal axis of the stipe explant, and they lead to the formation of a linear embryo (Mikuła et al. 2015b). By contrast, the zygote divides into four daughter cells, that initiate the four quarters of the zygotic embryo body of the fern (Johnson and Renzaglia 2008). The proliferation of zygotic initial cells depends on the orientation of the zygote in the archegonium and its position in relation to the gametophyte axis. The four quadrants will form root, leaf, foot and shoot apex of zygotic embryo, respectively (Johnson and Renzaglia 2008). Despite differences in the initiation of the somatic and zygotic embryos, later on their development is similar and they are able to develop into functional sporophytes (Mikuła et al. 2015b; 2018).

Plasmodesmata (PD) are recognized as essential regulatory elements in the development of multicellular plants (Otero et al. 2016). They facilitate the communication and transport of

materials, including signaling molecules, between plant cells (Zambryski 2004). The diameter and permeability of these symplasmic channels may be modified during cell/organ development or in response to external conditions and stimuli (Zambryski and Crawford 2000; Marzec and Kurczynska 2014). The functionality and role of plasmodesmata was widely explored in terms of organ development (Ding et al. 1999; Marzec and Kurczynska 2014; Otero et al. 2016), floral transition (Ormenese et al. 2000), dormancy induction in apical buds (Jian et al. 1997), embryo development during zygotic embryogenesis (Kim and Zambryski 2005; Stadler et al. 2005; Wróbel-Marek et al. 2017) and androgenesis (Wróbel et al. 2011). Many studies on symplasmic communication were based on the transport of tracers such as low molecular fluorochromes, fluorescent labeled dextrans, or green fluorescent protein (Crawford and Zambryski 2000; Kim et al. 2002; Wróbel et al. 2011; Marzec et al. 2014). Cell-to-cell transport of these substances allows to compare PD permeability for molecules of different size during developmental processes (Zambryski 2004). Although cell-to-cell communication is commonly accepted key that switches development from vegetative growth into embryogenic path, the issue is still poorly understood (Kurczynska et al. 2012). During induction of SE, the symplasmic communication is most often studied on the level of cellular changes. Of these, the cytolocalization of glucans, pectin epitopes or callose deposition are most frequently considered in response to osmotic challenges (Puigderrajols et al. 2001; Verdeil et al. 2001; You et al. 2006; Grimault et al. 2007). The analyses of symplasmic communication during SE with the use of fluorescent labels were performed only for Arabidopsis and showed the restriction in cell-to-cell movement through PD between parts of explants engaged in SE and those that were not involved in somatic embryo formation (Kurczyńska et al. 2007; Kulinska-Lukaszek and Kurczynska 2012; Godel-Jedrychowska et al. 2020).

The involvement of PD in regulation of cell differentiation and developmental patterns in ferns was proposed first many years ago, but it applies in particular to the gametophyte formation. It was shown that plasmolysis, which broke down the plasmodesmatal connections, led to abnormal branching of filamentous fern gametophytes and to re-differentiation of non-apical cells into apical type cells (Nakazawa 1963). This reaction was apparently caused by interrupted transmission of information determining the normal pattern of development (Drake et al. 1978). Functional PD are required for normal development of *Onoclea sensibilis* gametophyte (Tucker 1990). Disruption of symplasmic communication induces each cell of the gametophyte to become totipotent and to develop into an individual heart-shaped prothallus. Another example of the role of PD in fern development is provided by the discovery that symplasmic isolation of the apical cell of *Azolla* sp. root occurs by a reduction in the number

of PD as a result of successive divisions (Robards and Lucas 1990). This "dilution" of plasmodesmatal frequency is thought to control determinate growth pattern of the *Azolla* sp. root (Robards and Lucas 1990) and directions of apical cell divisions in the fern gametophyte (Holloway and Lantin 2002).

The aforementioned data encouraged us to ask whether the somatic-to-embryogenic transition in ferns is associated with changes in symplasmic communication. Such information would increase our knowledge on the role of PD from a physiological and evolutionary point of view, that is presently lacking for fern SE. In this study, we analyzed intercellular communication system that can cause epidermal cells of stipe explant to become embryogenic through a reduction in information exchange between explant cells. To that end, analyses of the distribution of fluorescent low-molecular-weight symplasmic tracers within the explants and early somatic embryos were undertaken to answer whether there is a correlation between changes in symplasmic communication and cell differentiation during fern SE.

Results

Fluorochrome distribution within initial explant

Epidermal and cortical cells of stipe explant were highly vacuolated and contained well-developed central vacuoles (Figs. 1a, b). In epidermal cells, the nucleus was located near the cell wall adjacent to the cortex (Fig. 1b). A thin layer of cytoplasm was located against the cell wall (Fig. 1a) and surrounded the nucleus (Fig. 1b). PD were present between epidermal cells as well as between epidermal and cortical cells (Fig. 1a).

In the initial explant, HPTS was present within the epidermal cells (Fig. 1c-e) and 2-3 cell layers of the cortex (Fig. 1e). Fluorochrome distribution in epidermal cells was uniform along the entire length of the explant (Fig. 1c). Inside the cell, fluorochrome was localized in the cytoplasm (layer present near the cell wall, around nucleus and plastids) and in cytoplasmic strands crossing vacuole (Fig. 1d). Fluorochrome was also detected in plastids (Fig. 1d). A similar pattern of fluorochrome distribution was observed following FDA treatment (data not shown). The results obtained indicate that in the initial stage, the whole explant is a single continuous symplasmic domain, at least in relation to epidermis and few cell layers of cortex directly below it.

Fluorochrome distribution within explant (6th day of culture)

On the 6th day of culture, no epidermal cell of stipe explant had divided yet. Their cytoplasm was dense and rich in numerous organelles (Fig. 2a). The central part of the cell was occupied

by the nucleus now. Their cell wall contained layers of electron-dense deposits and especially the middle lamella was strongly electron dense (Figs. 2b-d). In neighboring cortical cells, vesicles structurally resembling multivesicular bodies were present in the cytoplasm close to the vacuoles (Fig. 2d) or fused with plasmalemma indicating that unknown material is secreted into the periplasmic space (Fig. 2b).

Fluorescein was present only in some epidermal cells thus giving a patchy pattern of fluorochrome distribution (Fig. 2e). Fluorescein was not detected in cortical cells (Fig. 2e inset). A similar pattern of fluorochrome distribution was observed following HPTS treatment (Fig. 2f). These results indicate that the epidermis is no longer a single symplasmic domain. However, at this stage, it was not possible to determine which cells are symplasmically connected: the embryogenic or non-embryogenic.

Fluorochrome distribution within one-dimensional linear somatic embryos (8th and 10th day of culture)

The first stage of somatic embryo is a one-dimensional linear somatic embryo that is formed by the anticlinal divisions of single epidermal cell (Fig. 3a). Somatic embryos at this stage are characterized by a specific cell arrangement, one by one, which results in their characteristic linear shape. Usually embryo in this stage can be composed of up to 10 cells (Mikuła et al. 2015b).

At 8th day of culture, when the epidermal cells start to divide (Fig. 3a), the flow of fluorochrome between dividing cells and non-dividing cells of epidermis is stopped. HPTS provided to a single cell of the bi-cellular embryo moved to the second cell of the embryo but did not enter other cells of the explant (Fig. 3b, arrowhead points to the cell where fluorochrome was applied). This result was confirmed by FRAP technique (Figs. 3c-e). This experiment showed that after photobleaching (Fig. 3d), the fluorescein moved to the bleached cell from the non-bleached cell (Fig. 3e). Similar process was usually observed in tri-cellular embryos (Fig. 3f-h), but at this stage there were also embryos found where no movement of fluorescein between embryo cells took place after photobleaching (Fig. 3i-k). Both patterns of fluorochrome distribution were equally feasible as half of the examined embryos showed symplasmic coupling and the other half did not (Table 1). At 10th day of culture, following the next set of anticlinal cell divisions, one-dimensional, multicellular linear embryos were formed (Table 1). HPTS distribution between the non-dividing epidermal cells of the explant and cells of the embryo was still limited (Table 1). This data shows that a somatic embryo in early stage of development (bi-cellular embryo) represents a single symplasmic domain for at least low

molecular weight fluorochromes and does not exchange fluorochromes with neighboring explant cells. Moreover, it appears that with an increase in the number of embryo cells there is a reduction of fluorochrome exchange between them, which indicates that the symplasmic domain within the linear embryo have developed.

Fluorochrome distribution within two-dimensional linear somatic embryos (12th day of culture)

Following a series of anticlinal cell divisions, periclinal cell divisions of one-dimensional embryo began and the two-dimensional linear somatic embryo was formed (Fig. 4a). This somatic embryo has a linear shape consisting of a single layer of two-dimensionally arranged cells. As a result, the embryos protrude above the surface of the explant.

At 12th day of culture, when HPTS was applied to epidermal cells of explants, it was not distributed to neighboring cells of two-dimensional embryos (Fig. 4b; Table 1). This clearly indicates that at this stage of somatic embryo development a symplasmic communication between explant cells and the embryo is restricted. FRAP analyses performed on the somatic embryo (Fig. 4c-e) showed that after the photobleaching of fluorescein in one part of the embryo (Fig. 4d), the fluorochrome moved to it from adjacent embryo cells (Fig. 4e). The distribution of fluorescein within somatic embryos was not uniform (Figs. 4c, f), and lack of fluorochrome in few cells was observed in several embryos examined. The results obtained suggest that somatic embryos are symplasmically isolated from explant cells and cell-to-cell communication *via* PD is restricted in both directions: from somatic cells to embryo cells and vice versa (Table 1). The cytoplasm of somatic embryo cells was rich in numerous small vacuoles and starch grains located in amyloplasts (Figs. 4g-i). The cell wall between somatic embryo cells and other cells of the explant consisted of layers that differed in electron density (Fig. 4 h). Plasmodesmata were found in cell walls between embryo cells (Figs. 4g, i).

Fluorochrome distribution within spatial somatic embryos (16th day of culture)

Further numerous anticlinal, periclinal and oblique cell divisions of the embryo body cells resulted in the formation of the spatial somatic embryo (Fig. 5a). This somatic embryo consisting of a several layers of three-dimensionally arranged cells due to which its entire structure grows above the explant's surface horizontally and vertically.

After 16 days of culture, the embryo body consisted of four clearly delineated segments (Figs. 5a, d). HPTS distribution between the explant cells and somatic embryos was inhibited (Table 1). When fluorochrome was provided to cells of explant epidermis, it was never

translocated to somatic embryos (Fig. 5b). Furthermore, when fluorochrome was applied to the somatic embryo, it did not move to explant cells (Fig. 5c). It indicates a lack of fluorochrome exchange between cells following different developmental programs at the late stage of somatic embryo development. Additionally, restrictions in fluorescein movement between segments of the embryo were also clearly visible (Fig. 5c; Table 1). The somatic embryo, during segment differentiation became isolated from the external environment (Figs. 5d, e) and from the explant cells (Figs. 5d, f) by a cell wall that was composed of layers differing in electron translucency. This stratification was visible for both embryo cells and cortical cells (Fig. 5g). Numerous vesicles were present in the periplasmic space of these cells. The PD were present between cells of the somatic embryos (Fig. 5h). The cytoplasm of somatic embryo cells was rich in endoplasmic reticulum structures, ribosomes, dictyosomes, mitochondria and small vacuoles (Figs. 5e, f, h). In developing somatic embryo, diversity in cell size, level of vacuolization and starch content was observed (Fig. 5i).

Discussion

The formation of a complex multicellular organism from a single explant cell cultured under *in vitro* conditions is one of the most amazing processes in plant biology. Induction of this process demands activation of a new morphogenetic developmental program in explant cells. Some research indicates that changes in symplasmic communication may be of key importance for these processes (Ding et al. 1999; Marzec et al. 2014; Otero et al. 2016). Additionally, research concerning SE is complicated due to often occurring indirect and side pathways of somatic embryo differentiation and multicellular origin of somatic embryos as sometimes several types of tissues may be involved. The model developed for the tree fern *C. delgadii* allows us to follow the transition of somatic cells into embryogenic ones. Furthermore, the subsequent formation of somatic embryo passing through the characteristic morphological stages, i.e. a series of anticlinal divisions and the formation of four segments of the embryo body, can be tracked down with high precision.

Induction of somatic embryogenesis

The studies presented here indicate that during SE of the tree fern *C. delgadii* lack of exchange of substances, that have similar physico-chemical properties to the fluorochromes used, occurred. Thus, explant cells are symplasmically isolated during the acquisition of embryogenic competence.

In many works, plasmolysis is an effective factor inducing the changes in cell fate leading to an increase of the ratio of somatic embryo formation (Lou and Kako 1995; Choi and Soh 1997; Puigderrajols et al. 2001; Verdeil et al. 2001; You et al. 2006; Karami et al. 2006; Grimault et al. 2007). Plasmolysis disrupts the cellular interconnections between explant cells allowing more cells to undergo re-programing and start to develop into somatic embryos. Published reports demonstrate that the exchange of information through PD may influence plant development and cell differentiation. Cells symplasmically connected by PD are characterized by a similar frequency and direction of divisions and follow the same developmental program (Marzec and Kurczynska 2014). Spatio-temporal changes in symplasmic communication result in symplasmic domain formation in which the cells follow different developmental programs (Ehlers et al. 1999). The data presented here indicate that in the case of fern SE, relationships between symplasmic isolation and changes in cell fate, including re-establishment of cellular totipotency, also occur. These results are new for fern SE and are consistent with data obtained from angiosperm plants (Kulinska-Łukaszek and Kurczyńska 2012; Kurczynska et al. 2007). Thus, our results seem to confirm that the interruption of cell-to-cell communication stimulates the reprogramming of somatic cells into embryogenic state and induces SE. At present, we are not able to explain the mechanism of this interruption. However, we assume that it may be based on a series of physiological rearrangements that the explant undergoes at an early stage before the cell divisions leading to somatic embryo formation occur (Grzyb et al. 2017). Among them, a sudden increase in the concentration of soluble sucrose is one of the first and the most discernible reactions that may switch on the developmental program which allows certain epidermal cells to regain their potential for SE.

Somatic embryo development

During the development of somatic embryos in *C. delgadii* explants there is a temporary loss of communication between cells of the embryo body. Changes in PD permeability appear first at the tri-cellular embryo stage. An interruption of cell-to-cell communication does not interfere with the occurrence of further divisions, which are only transverse to the axis of the explant. After 8-10 divisions, restrictions in symplasmic communication are still present in the embryo. At this point, reduced cytoplasmic flow can lead to the divisions occurring in a different orientation and with variable frequency. Contrary to our results, in the early zygotic embryo of Arabidopsis, all cells form a single symplasmic domain until the early torpedo stage (Kim et al. 2005).

Changes in the direction of cellular divisions in the one-dimensional linear somatic embryo of *C. delgadii*, as well as frequency of divisions, lead to the formation of four segments, each being separate symplasmic subdomain. Our study showed that symplasmic communication is restricted between the embryo segments, but cells of the same segment are symplasmically interconnected. Similarly, Kim and co-workers (2002) showed that the occurrence of symplasmic domains in the zygotic embryo of Arabidopsis precedes the differentiation of organs i.e. roots and leaves. However, only transport of large molecular weight tracer (10 kD fluorescent dextran) was limited whereas the smaller molecular weight tracer (HPTS) was transported in all stages of embryogenesis. We speculate that symplasmic isolation is extremely important in the process of differentiation into 4 segments of the somatic embryo in ferns. Although at present we cannot yet indicate which organ becomes differentiated from each segment of the *C. delgadii* embryo, we can only assume that the most actively dividing segment (such as the region 2 in Fig. 5i) will give rise to the first leaf. Our previous studies showed that this segment of the zygotic embryo of *C. delgadii* develops faster than the others (Mikuła et al. 2018). It seems that the difference in the intensity of cellular divisions may result from the unequal transport of hormones and other molecules or signals between particular segments of the embryo body. In plant embryogenesis, positional information establishes the overall body plan and lineage-dependent cell fate specifies local patterning. Taking Arabidopsis as a model, researchers emphasize that the auxin signaling (Berleth and Chatfield 2002) in combination with specific genes products (Laux et al. 2004) regulates the formation of the basic apical–basal body pattern in early embryos. Moreover, movement of auxin through PD was elucidated by studies of Arabidopsis mutant *gsl8*, which has increased plasmodesmal permeability (Han et al. 2014). Thus, our studies show PD as dynamic intercellular channels for the transport of embryo-specific factors regulating somatic embryo development.

Considering that somatic embryo cells become symplasmically isolated from explant early upon their induction, and the isolation persists at least until the embryo reaches the spatial stage, the question remains of how the embryo is nourished. Bell (1986) showed that starch is accumulated abundantly in the egg cells of a number of ferns. In accordance, our studies revealed the numerous starch grains accumulating in some cells of the explant epidermis, and also in the somatic embryos composed of several cells. This may provide an energy reserve allowing somatic embryo development in the absence of symplasmic connection with the explant. In young zygotic embryo, the foot expands and differentiates to facilitate transport of nutrients from the gametophyte to the developing embryonic organs (Johnson and Renzaglia

2008). Although some parts of the four-segment somatic embryo of *C. delgadii* contain more starch deposits than others, we were not able to document development of placenta.

Conclusion

1. The somatic-to-embryogenic transition is preceded by restrictions in distribution of low-molecular weight fluorochromes between cells of the initial explant. Thus, we assume that symplasmic isolation of selected explant cells is a prerequisite for reprogramming of cell development and initiation of the morphogenetic response.
2. The bi-cellular somatic embryo is a single symplasmic domain.
3. The restrictions in the fluorochrome flow appear first in tri-cellular embryos and the embryo ceases to be a single symplasmic domain.
4. In fern somatic embryo, symplasmic subdomains are formed that correspond to the four segments of embryo body. Thus, we conclude that changes in the plasmodesmatal connections are pre-requisite for regular embryo development.
5. The data presented provide a novel view on fern somatic embryogenesis as a process regulated by symplasmic communication.

Materials and methods

Plant material and culture conditions

Sporophytes that had developed 2 or 3 leaves were used as a source of explants. Plant material was collected from the youngest fronds of 5-month-old *in vitro*-grown sporophytes maintained in darkness. The plant material was cultured on a hormone-free agar medium containing half-strength macro- and micronutrients basal MS medium (Murashige and Skoog 1962) supplemented with vitamins and 1% sucrose (Mikuła et al. 2015a).

To carry out microscopic analyzes the plant material was successively collected after 6, 8, 10, 12, 16 days of culture. Stipe explants, about 2.5 mm in length, were freshly cut off from the first fronds of donor sporophytes and used as initial explants. Five to twenty explants were analyzed for each time point.

Fluorescent probes

Symplasmic communication was investigated using low molecular weight fluorochromes: 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS; Sigma-Aldrich, H1529) and fluorescein. HPTS is a membrane impermeable fluorochrome, and a recommended symplasmic tracer (Wright and Oparka, 1996). Thus, plasma membrane was injured with a

microcapillary to allow its uptake into cytoplasm (see below). Fluorescein was delivered to cells in the form of fluorescein diacetate (FDA; Sigma-Aldrich, F7378). FDA is a non-fluorescent, membrane permeant compound. After uptake into cytoplasm, cell esterases cleave off acetate groups and release fluorescein into the cytoplasm. Fluorescein is fluorescent and membrane impermeant (Wright and Oparka 1996). In experiments, HPTS fluorescence was more stable than fluorescein fluorescence, so it could be observed for a long time without bleaching. As fluorescein was very sensitive for bleaching it was chosen for FRAP experiments (Fluorescence Recovery After Photobleaching; described below). Both fluorochromes (HPTS and fluorescein) upon uptake into cytoplasm move from one cell to another only *via* plasmodesmata, thus were used in the studies of symplasmic communication (Gisel et al. 2002; Wróbel et al. 2011; Maule et al. 2013; Milewska-Hendel et al. 2019). For symplasmic communication analyses, HPTS solution (5 mg/ml in water) and FDA solution (5 mg/ml stock FDA in acetone diluted 1:10 in water) were used.

Application of fluorochromes

Analysis of symplasmic communication was conducted on initial explants and explants cultured for 6-16 days (Fig. 6). The initial explants were incubated for 60 min. in a HPTS (or FDA) solution immediately after excision from the sporophyte. Cultured explants were incubated in 0.1 mM aqueous solution of 2-deoxy-D-glucose (DDG; Sigma-Aldrich, D6134) for 30-60 min. to prevent callose deposition. Explants were then immersed in a HPTS (or FDA) solution and punctured randomly (by hand, under a stereomicroscope) with a microcapillary of outer diameter between 7 and 10 μm. Subsequently, both ends of explants were cut off and explants were incubated in the fluorochrome solution for 60 min. After incubation, the plant material was rinsed with demineralized water to wash out excess of fluorochrome and then analyzed under a confocal laser scanning microscope (CLSM) as described below.

Viability tests

Cell viability tests were performed with 0.01 μg/ml solution of propidium iodide (PI; Sigma-Aldrich, P4170) and 0.01% (w/v) solution of Evans blue (Sigma-Aldrich, E2129) dyes. Plant material was incubated in PI solution after FDA (or HPTS) treatment. Thus, it was possible to collect images of fluorescein (or HPTS) and PI simultaneously (see below). Explants were stained with Evans blue solution after CLSM analysis, because this dye is used for bright field microscopy and its hue is not visible in the "bright field" mode of CLSM. Dead cells in explants were not analyzed.

Confocal laser scanning microscopy

For direct, noninvasive, serial optical sectioning of intact, thick, living samples a confocal laser scanning microscope FV 1000 (Olympus) which is built onto IX81 inverted fluorescence microscope equipped with a multi-band argon laser was used to excite fluorescence of fluorochromes. HPTS and fluorescein were excited with 488 nm wavelength and emission was collected at 500-530 nm. In all analyses, laser power was set at 5-10% for HPTS excitation and 2-4% for fluorescein excitation. Voltage on photomultipliers (PMTs) was set at 530-700 V for both fluorochromes. PI was excited with 543 nm wavelength and emission was collected at 555-625 nm (laser power: 23-25%, PMT: 680-700V).

FRAP experiments were performed on plant material incubated in FDA. Firstly, the region of interest (ROI) was selected using CLSM software (FluoView 1000) and it was irradiated with 405 nm laser (laser power set at 60-90%) for 30-60 s to bleach the fluorescein. Next, time series images were collected to check whether the fluorochrome had returned to the bleached area/cells (excitation 488 nm). The mean value of fluorescein fluorescence was measured in the bleached area just after bleaching and during next 3-5 min. If the mean value of fluorescence rose by 20% (or more) it was assumed that the recovery occurred. Time series analyses were carried out for 3-5 min. because prolonged acquisition of images resulted in a reduction in fluorescein fluorescence intensity.

Before collecting data, autofluorescence of explants and embryo cells was checked for all plant material. For analyses of symplasmic fluorochrome movement, CLSM settings were used when no autofluorescence signal was collected by photomultipliers in control unstained samples, with an exception of chlorophyll autofluorescence. However, emission wavelengths of chlorophyll autofluorescence (630-700 nm) did not overlap with those of HPTS and fluorescein (500-530 nm) and chlorophyll autofluorescence could be examined simultaneously with both fluorochromes.

Digital CLSM images were prepared for publication using FluoView and ImageJ software. Data for fluorochrome movement between explant and somatic embryo cells, and within somatic embryo segments are given in Table 1.

Transmission (TEM), scanning (SEM) electron microscopy and light microscopy analyses

Plant material was prepared as described previously (Domżalska et al. 2017). The explants were fixed in a mixture of 2.5% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2; room temperature; 24 h). Next, the

samples were post-fixed in 2% osmium tetroxide in 0.05 M cacodylate buffer at 4 °C for 6 h and dehydrated in a graded ethanol series (30, 50, 70, 90, 96 and 3 times 99.8%) for 2 h at each concentration, followed by propylene oxide substitution. The samples were infiltrated with graded series of Epon epoxy resin (Sigma) mixtures for 48 h in total and polymerized at 65 °C for 16 h. For TEM, ultrathin sections (90 nm thick) were cut with a Leica EM UC6 ultramicrotome and collected onto carbon-coated copper grids (300 mesh). They were contrasted in a saturated solution of uranyl acetate dissolved in 50% (w/v) ethanol (30 min) and 0.04% (w/v) lead citrate (30 min). Specimens were examined with a FEI 268D 'Morgagni' (FEI Corp., Hillsboro, USA) transmission electron microscope operating at 80 kV equipped with an Olympus-SIS 'Morada' 11 Mpix digital camera or with a Jeol JEM-3010 (Jeol, Tokyo, Japan) high resolution electron microscope (accelerating voltage 300 kV) equipped with a Gatan 2k × 2k Orius TM 833 SC200D CCD camera. For TEM analysis, at least 20 sections through the all tissue layers of the explant were examined.

For light microscopy, 2 μm-thick sections were collected onto microscope slides. Samples were stained with 0.1% (w/v) toluidine blue in 1% (w/v) borax. The semi-thin sections were observed using a light Olympus Vanox microscope.

For SEM analyses, non-fixed plant material was examined with the FEI Quanta 200 environmental scanning electron microscope (ESEM) at a relative humidity of up to 100%, and at a vacuum lower than 10^{-4} Pa.

Founding

This work was supported by the Polish National Science Centre (NCN) grant no. 2017/27/N/NZ3/00434.

Acknowledgments

We thank Anna Milewska-Hendel for providing a workshop on TEM techniques for Małgorzata Grzyb.

Disclosures

Conflicts of interest: No conflicts of interest declared.

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Tables

Table 1. Fluorochrome movement between somatic embryo and explant, and within somatic embryo (Se).

Figures

Figure 1. Microscopic analysis and symplasmic tracer distribution in the initial stipe explants. **a:** Cross-section of highly vacuolated epidermal and cortical cells. **b:** The epidermal cell contains thin layer of cytoplasm and nucleus located near the cell wall neighboring cortex (longitudinal section of explant). **c:** HPTS accumulation (green) in the epidermal cells of the initial explant (top surface view of the middle part of the explant is shown). **d:** HPTS accumulation (green) in the thin peripheral cytoplasm next to the cell wall (CW*), around the nuclei (N*) and around plastids (P*) of epidermal cells. **e:** Orthogonal projection of the same explant as depicted on figure **d**.

c-e: Explant incubated in aqueous solution of HPTS for 60 min. TEM (**a**, **b**) and CLSM (**c-e**) images. Bars = 5 μm (a, b), 100 μm (c), 25 μm (d), 50 μm (e)

Am, amyloplast; C, cytoplasm; CW, cell wall; Cx, cortex; Ep, epidermis; M, mitochondrion; N, nucleus; Nl, nucleolus; V, vacuole; St, stoma; Red arrows- plasmodesmata

Figure 2. Microscopic analysis and symplasmic tracer distribution in 6-day old explants.

a: Epidermal cells have dense cytoplasm, large nucleus, numerous mitochondria and amyloplasts, and vacuoles with electron-dense granules. Cortical cell has electron lucent cytoplasm (cross-section of explant). **b, c, d:** Ultrastructural details of the cell walls between epidermis and cortex (**b, d**) and between two epidermal cells (**c**). Vesicles probably directed to periplasmic space (**b**) or to the vacuole (**d**). **e, f:** Patchy distribution of fluorescein (green) (**e**) and HPTS (green) (**f**) in epidermal cells of explant incubated in aqueous fluorochrome solution for 60 min (top surface views of the explant). Insets on **e** and **f** are orthogonal projections of samples depicted on corresponding figures proofing the presence of fluorochrome only in epidermal cells.

TEM (**a-d**) and CLSM (**e**, **f**) images. Bars = 5 μm (a), 2 μm (b, c), 1 μm (d), 100 μm (e, f)

Am, amyloplast; C, cytoplasm; CW, cell wall; Cx, cortex; Ep, epidermis; G, electron-dense granules; ML, middle lamella; M, mitochondrion; N, nucleus; Nl, nucleolus; P, plastid; Pc, paracrystal bodies; ER, endoplasmic reticulum; V, vacuole; Vc, vesicles; Arrowhead – point of fluorochrome application

Figure 3. Histology and fluorochrome distribution in one-dimensional linear somatic embryos induced from single epidermal cells of stipe explants after 8 (**a-e**) and 10 days of culture (**f-k**). **a:** Longitudinal section of bi-cellular somatic embryo with well distinguishable cell wall between divided cells (arrow). **b**: HPTS (green) accumulation in bi-cellular embryo and autofluorescence of chlorophyll (red) in adjacent epidermal cells. **c-k:** FRAP analysis conducted on bi-cellular (**c-e**) and tri-cellular embryos (**f-k**). Presence of fluorescein in embryo cells before FRAP (**c, f, i**), immediately after photobleatching (**d, g, j**), and after 5 minutes of recovery after photobleaching (**e**, **h, k**); top surface views of somatic embryo.

Light microscopy after toluidine blue staining (**a**) and CLSM (**b-k**). Bars = 20 μm (a), 100 μm (b), 50 μm (c-k)

Se, somatic embryo; Cx, cortex; Arrowhead – site of fluorochrome application; Red line indicates the bleached cell; White dotted line indicates cell walls of examined cells of somatic embryo

Figure 4. Microscopic analysis and fluorochrome distribution in two-dimensional linear somatic embryos at 12th day of the culture.

a: Multicellular somatic embryos induced from single epidermal cells after series of anticlinal and periclinal cell divisions (longitudinal section of tangent of the explant surface). **b:** HPTS accumulation (green) occurs only in injured and neighboring epidermal cells of explant after incubation for 60 min. in fluorochrome solution and is absent from somatic embryo cells. The red autofluorescence of chlorophyll in the cells of somatic embryos is only visible (embryos are at the same stage of development as embryos depicted on figure **a**). **c-e:** FRAP analysis (side surface view of somatic embryo). The area where fluorochrome was bleached is indicated by red line. Fluorescein fluorescence in the embryo before FRAP (**c**), immediately after photobleaching (**d**), and after 5 min. of recovery after photobleaching (**e**). **f:** Diverse distribution of fluorescein in the embryo body (top surface view of somatic embryo). **g-i:** Ultrastructure of multicellular somatic embryo; the transverse section shows two embryo cells rich in cytoplasm and organelles connected with numerous plasmodesmata (red arrows). **h, i:** enlargements of areas marked with rectangles on figure **g**. **h:** Part of the cell wall between somatic embryo and explant. **i:** Part of cell wall within the embryo.

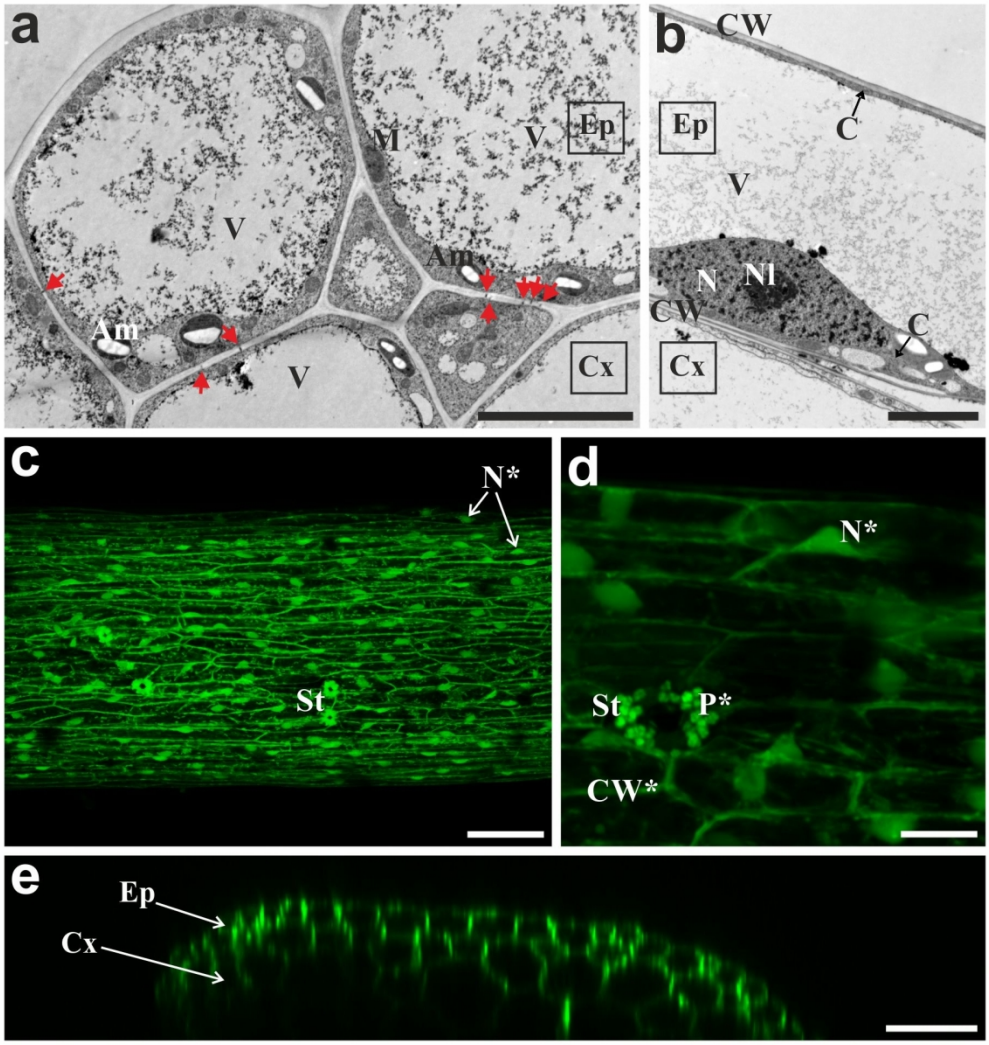
Light microscopy after toluidine blue staining (**a**), CLSM (**b-f**) and TEM (**g-i**). Bars = 20 μm (a), 100 μm (b), 50 μm (c-f), 10 μm (g), 2 μm (h, i)

Am, amyloplast; Cx, cortex; Se, somatic embryo; CW, cell wall; G, electron-dense granules; M, mitochondrion; Pc, paracrystal bodies; V, vacuole; Red arrows- plasmodesmata;

Arrowhead –site of fluorochrome application; Red line indicates the bleached cells. White dotted line indicates cell walls and outlines somatic embryos.

Figure 5. Microscopic analysis of spatial somatic embryos at 16th day of the culture.
a: SEM image of the embryo with four segments (dashed arrows point to borders between segments). **b:** HPTS accumulation (green) in some epidermal cells of explant near its application site (arrowhead), and autofluorescence of chlorophyll (red) in the cells of somatic embryos and other cells of the explant. **c:** Localization of fluorescein is restricted to specific segment of embryo after fluorochrome application to the cell marked with an arrowhead (side surface view of somatic embryo). **d:** Longitudinal section of somatic embryo composed of four segments. Arrows point to borders between segments. Selected areas (indicated by letters e-h next to squares) were analyzed by TEM. **e:** Outer periclinal wall of the embryo and a fragment of cytoplasm with plastids, endoplasmic reticulum, ribosomes and vesicles. **f:** Anticlinal wall between two cells of somatic embryo and inner periclinal wall between somatic embryo and cortex cell with well distinguishable middle lamella as an electron opaque layer. Undulated plasmalemma indicates intensive processes of exocytosis as confirmed by the presence of numerous dictyosomes of Golgi apparatus. **g:** Deposition of a numerous vesicles in the periplasmic space. **h:** Cell wall between segments of somatic embryo. **i:** Longitudinal section of somatic embryo composed of embryo regions with cellular heterogeneity: 1 – large and vacuolated cells; 2 – small and abundantly dividing cells; 3 – medium-sized cells; 4 – large cells being rich in starch (arrows).
SEM (**a**), CLSM (**b, c**) light microscopy after toluidine blue staining (**d, i**), and TEM (**e-h**). Bars = 100 μm (a, c), 500 μm (b), 50 μm (d), 0.5 μm (e), 2 μm (f), 1 μm (g), 5 μm (h), 20 μm (i)
Am, amyloplast; CW, cell wall; CW, cell wall between embryo segments; Cx, cortical cell; ER, endoplasmic reticulum; Ex, explant; GA, dictyosomes of Golgi apparatus; ML, middle lamella; M, mitochondrion; PM, plasma membrane; PS, periplasmic space; Se, somatic embryo; V, vacuole; Vc, vesicles; Red arrows- plasmodesmata; Arrowhead –site of fluorochrome application*

Figure 6. Scheme of fluorochromes application to initial stipe explants and to explants cultured for 6-16 days.
DDG, 2-deoxy-D-glucose; FDA, fluorescein diacetate; HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt; Marked in black - two ends of explant browning during the culture; Marked in green – fluorochrome solution



Microscopic analysis and symplasmic tracer distribution in the initial stipe explants.

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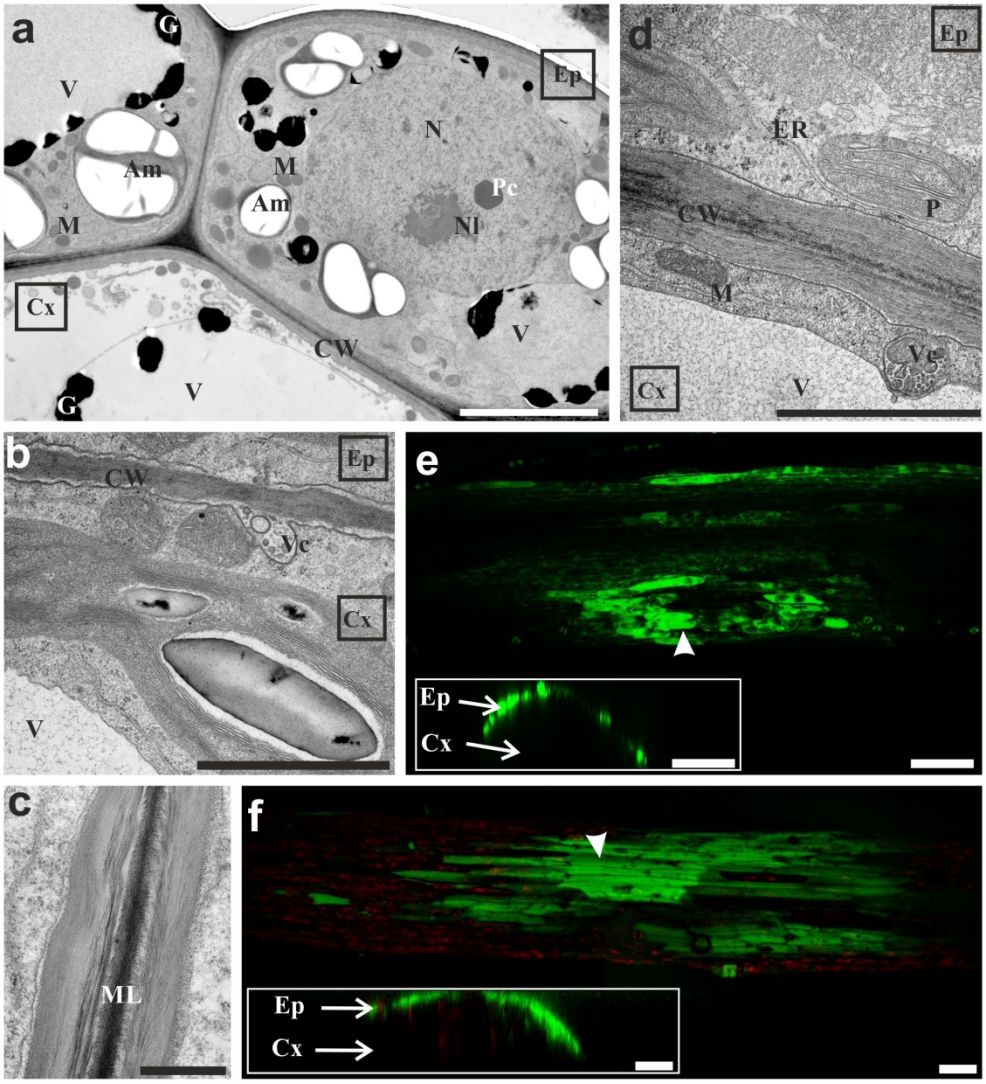

Microscopic analysis and symplasmic tracer distribution in 6-day old explants.

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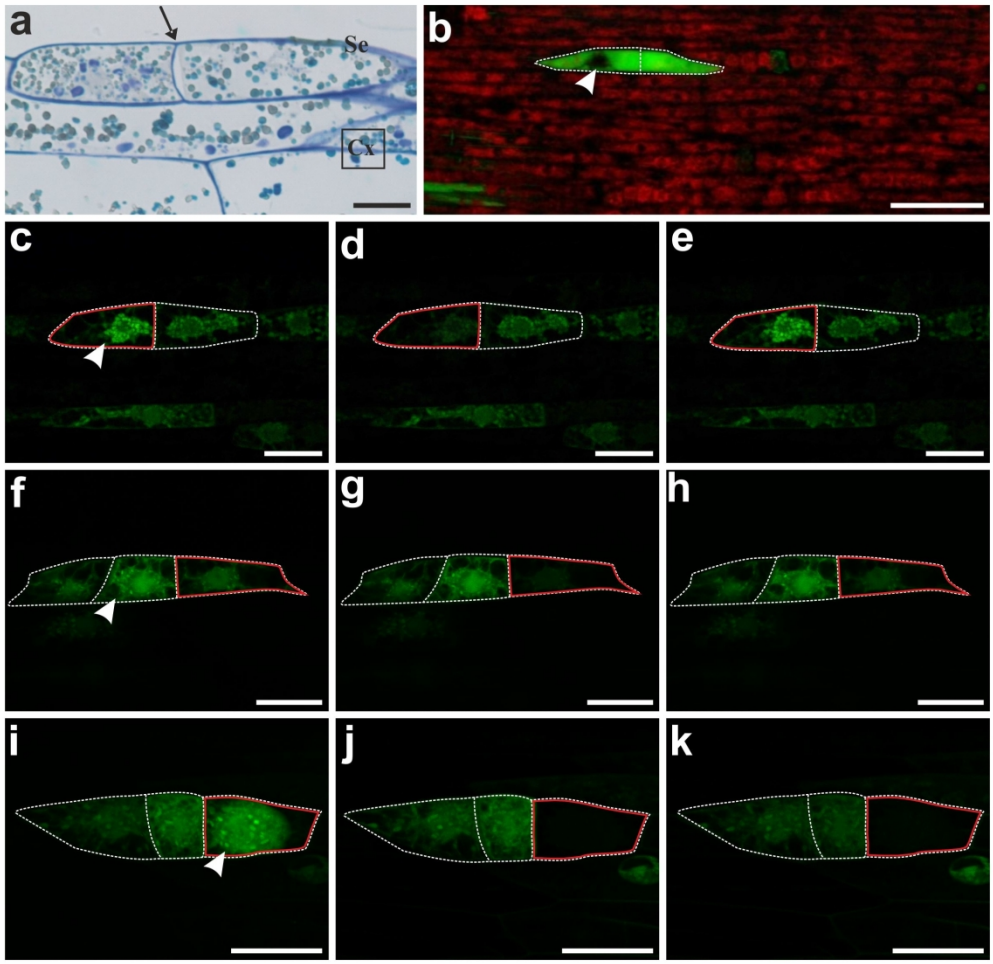

Histology and fluorochrome distribution in one-dimensional linear somatic embryos induced from single epidermal cells of stipe explants after 8 (a-e) and 10 days of culture (f-k).

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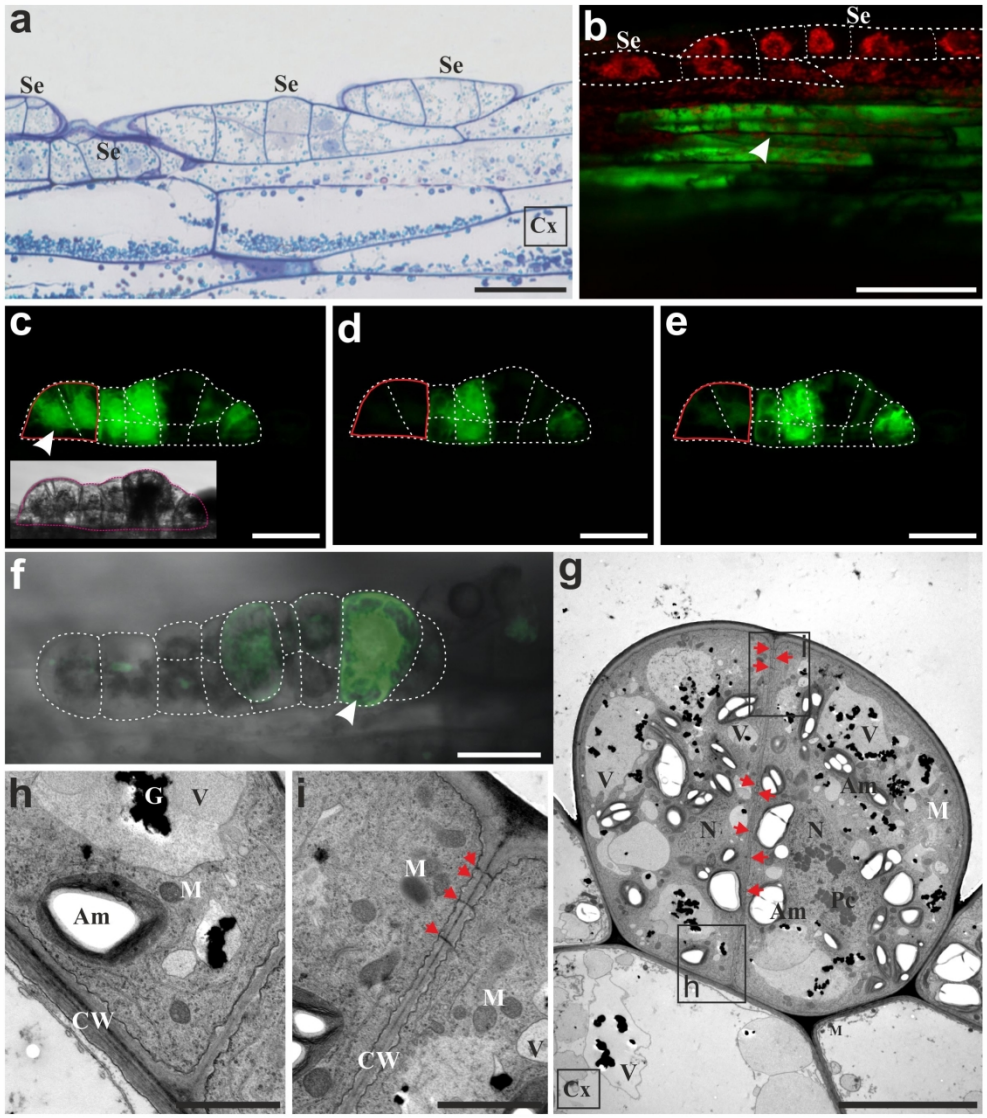

Microscopic analysis and fluorochrome distribution in two-dimensional linear somatic embryos at 12th day of the culture.

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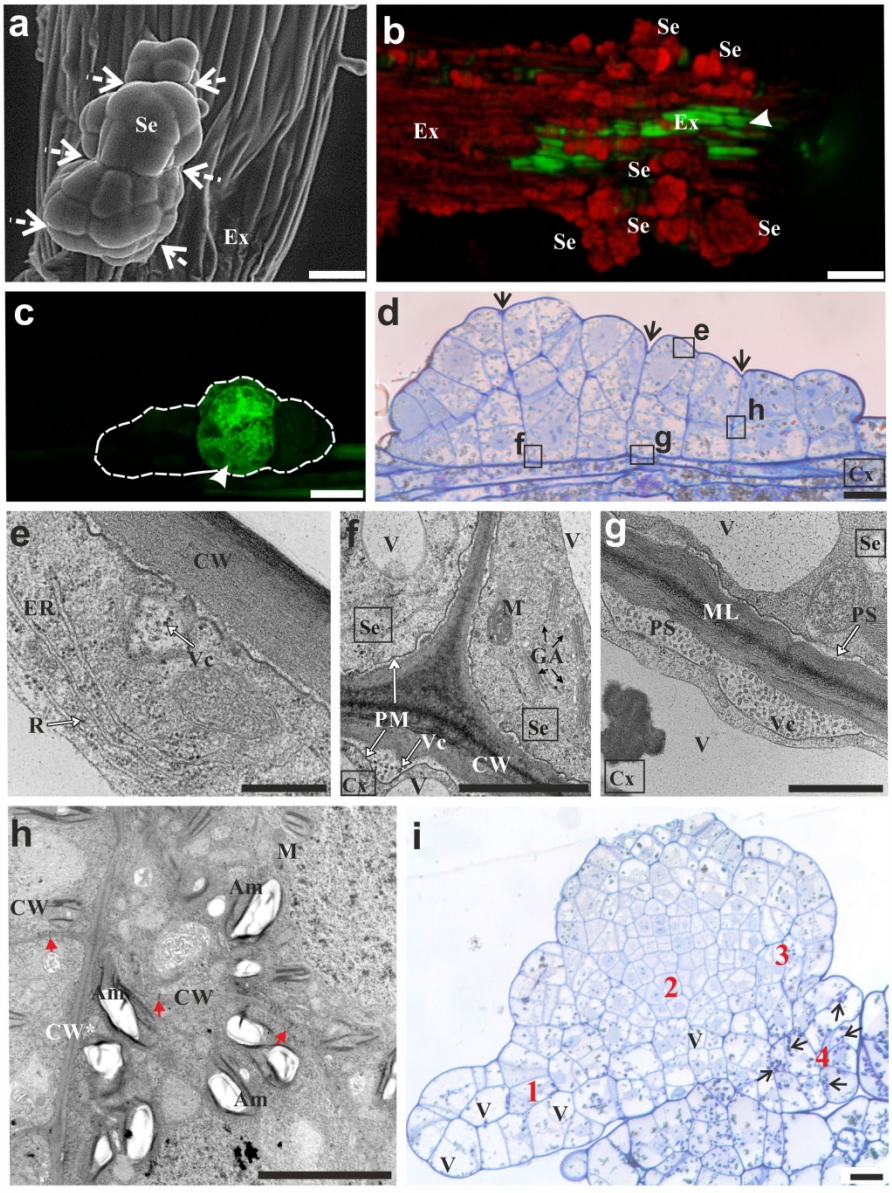
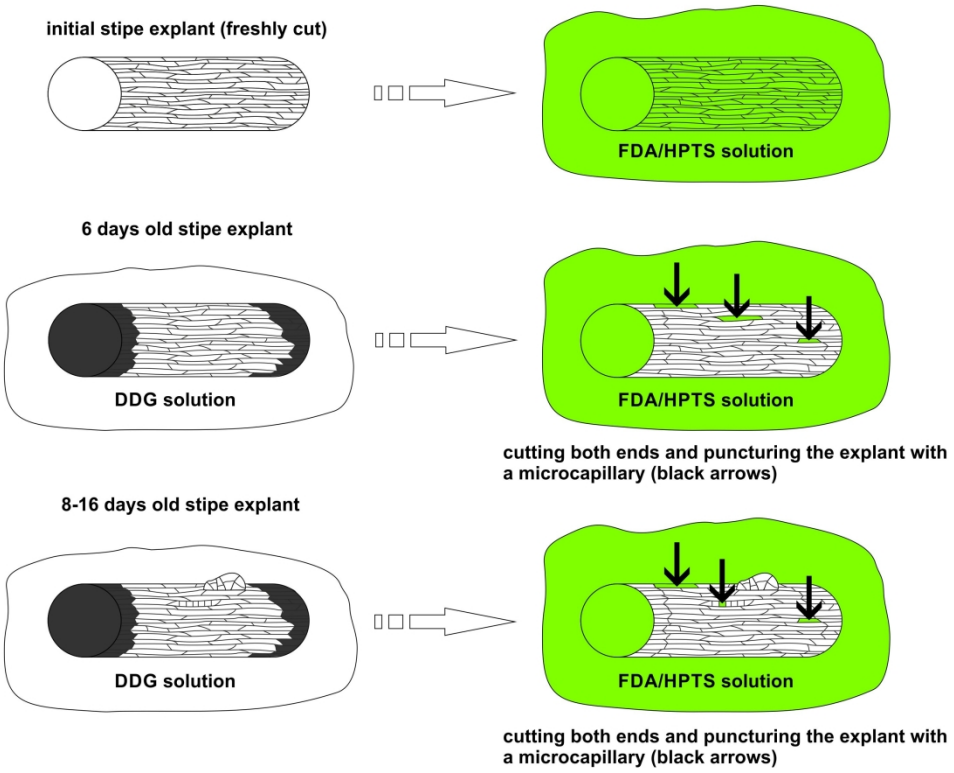


Microscopic analysis of spatial somatic embryos at 16th day of the culture.

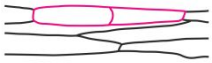
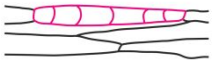
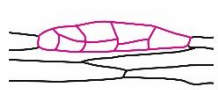
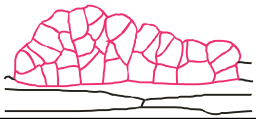
202x267mm (300 x 300 DPI)



Scheme of fluorochromes application to initial stipe explants and to explants cultured for 6-16 days.

264x208mm (300 x 300 DPI)

Table 1. Fluorochrome movement between somatic embryo and explant, and within somatic embryo (Se).

Developmental stage of somatic embryo	Explant→Se	Se→Explant	Between embryo cells	
One-dimensional linear embryo				
2-cell embryo 	3[a]/8[b]	1/10	10/11	
3-cell embryo 	n/a	1/9	4/8	
6-cell embryo 	1/8	0/11	5/10	
Two-dimensional linear embryo 	1/15	0/15	7/15	
			within segment	between segments
Spatial embryo 	3/20	0/20	10/10	0/10

a- the number of cases when the fluorochrome is distributed between the analyzed areas
b- the number of analyzed cases
n/a-the cases which have not been analyzed

Plant Cell, Tissue and Organ Culture (PCTOC) (2019) 136:221–230
https://doi.org/10.1007/s11240-018-1507-5

ORIGINAL ARTICLE

Explant type and stress treatment determine the uni- and multicellular origin of somatic embryos in the tree fern *Cyathea delgadii* Sternb.

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Received: 2 May 2018 / Accepted: 7 October 2018 / Published online: 11 October 2018

Abstract
Cyathea delgadii is the first fern species for which somatic embryogenesis (SE) has recently been described. With this discovery, a new experimental model for exploration of SE was found. In this study, the effect of explant type (stipe and internode), length and diameter of the explant-donor frond, and stress treatment (by sucrose and air desiccation) on somatic embryo origin and SE efficiency was studied. In control culture, somatic embryos originated from single cells of stipe explants, whereas those induced on internodes were of multicellular origin. Although the activation of cell divisions was more abundant and the formation of somatic embryos occurred earlier in a culture of internodes than of stipe explants, the morphogenic capacity of internodes was much smaller. On their surface, the groups of competent cells formed protuberances that give rise to only three somatic embryos per internode. In contrast, almost 92% of stipe explants that were excised from the first frond, measuring 10 mm in length and less than 300 µm in diameter, produced an average of 21 somatic embryos. Stipes treated with sucrose were not able to SE. More than fourfold increase in SE efficiency was obtained on internodes by their treatment with 0.4 M sucrose for 45 min. It was achieved by changing the pathway of somatic embryo differentiation from multicellular to unicellular. These results provide an excellent basis for further research on the mechanism of SE induction associated with a single- and multi-cell proliferation, especially that both types of the embryogenic pathway can occur on the same hormone-free medium.

Keywords Air desiccation · Hormone-free medium · Internode and stipe explants · Microscopic analysis · Somatic embryogenesis · Sucrose treatment

Introduction

Somatic embryogenesis (SE) is a remarkable expression of cellular totipotency, by which plant somatic cells undergo reprogramming and acquire the competence to assume a new developmental fate. In this process, somatic embryos may develop directly from cells of explant, or indirectly with an intermediate callus phase (Williams and Maheswaran 1986). In direct SE, depending on the relationship between neighbouring cells within the explant, the embryos are able to initiate from a single cell (Choi et al. 1998; Mikuła et al. 2015b) or groups of competent cells (Choi and Soh 1995; Taylor and Vasil 1996; Choi et al. 1998; Puigderrajols et al. 2001; Corredoira et al. 2006). Although SE has been successfully achieved in numerous species of seed plants, only a few published reports describe this phenomenon in monilophytes, and all these studies were performed on the tree fern *Cyathea delgadii* Sternb. (Mikuła et al. 2015a, b; Domżalska et al. 2017; Grzyb et al. 2017, 2018). These investigations demonstrated that somatic embryos of *C. delgadii* are formed directly from single epidermal cells. Embryogenic cultures of ferns, that represent the closest living relatives of spermatophytes (Pryer et al. 2001), promise to be useful in the study of some fundamental problems relating to somatic embryo initiation and development (Domżalska et al. 2017; Grzyb et al. 2017, 2018). However, a better understanding of factors that control the somatic embryo origin and improve the efficiency of SE in ferns is still required.

The pathway and effectiveness of somatic embryo differentiation depends on various factors (Gaj 2004). The source

Communicated by M. I. Beruto.

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and physiological state of the explant appears to be the main initiator of direct SE (Williams and Maheswaran 1986; Merkle et al. 1995; Choi et al. 1998). Generally, somatic embryos can be induced from a range of explants and their highly differentiated cells, such as those of the leaf (Wang and Bhalla 2004; Chung et al. 2007), root (Iantcheva et al. 2005), glandular trichomes (Kim et al. 2007) or even fully differentiated stomatal guard cells (Chen and Hong 2012). However, immature and young plant material, such as zygotic and somatic embryos, or parts of seedlings, appears to be more responsive to in vitro treatments (Williams and Maheswaran 1986; Merkle et al. 1995). Its cells require minor gene reprogramming and hence, direct SE can be readily induced from these (Merkle et al. 1995; Karami et al. 2009). The initiation of SE can occur simultaneously along unicellular and multicellular pathways on the same explant, as it was demonstrated for zygotic embryo cultures of white clover (Maheswaran and Williams 1985), pearl millet (Taylor and Vasil 1996) and pineapple guava (Canhoto and Cruz 1996; Canhoto et al. 1996). Alternatively, both pathways of SE can be induced independently of each other by using various types of initial explants (Choi et al. 1998; Maximova et al. 2002) or plant growth regulators (PGRs) (Lee et al. 1990; Choi and Soh 1995; de Almeida et al. 2012). The number of explant cells involved in somatic embryo production is important in connection with the genetic chimerism of regenerated plants. Therefore, unicellular origin of embryo may be more desirable than multicellular one. The embryogenic competence of in vitro-cultured somatic cells can also be stimulated by high osmotic pressure. This treatment has been shown to greatly enhance somatic embryo production (Kamada et al. 1993; Ikeda-Iwai et al. 2003; Karami et al. 2006; You et al. 2006; Mikuła et al. 2011a, b) and germination (Attree et al. 1991).

Somatic embryogenesis in ferns was first reported for *C. delgadii* as the most promising tissue culture technology to date for the large-scale clonal propagation of these plants (Mikuła et al. 2015b). In order to efficiently induce somatic embryos in this species on hormone-free medium, the role of different endogenous and exogenous factors should be investigated. Our previous work demonstrated that the source of plant material (etiolation and age of donor plants, length of initial explants), as well as physical (photoperiod, darkness) and chemical (sucrose and mineral salt concentrations in the medium) factors affected the efficiency of SE (Mikuła et al. 2015a). The basis for the induction of SE in *C. delgadii* is an appropriate hormonal balance in explant cells established by etiolation of donor plantlets (Grzyb et al. 2017). We also improved the current understanding of physiological and molecular processes involved in the induction and expression of early SE in this species (Grzyb et al. 2018; Domżalska et al. 2017). Our previous achievements have been summarised in the latest review article (Mikuła et al. 2018). The present paper focuses on the effect of explant type, length and diameter of explant-donor frond and stress treatment on the pathway and efficiency of SE in *C. delgadii*.

Materials and methods

Plant material and growth conditions

The source of plant material were somatic embryo-derived sporophytes of *C. delgadii* cultured on Murashige and Skoog's (1962) medium containing half-strength of macro- and micronutrients, a full complement of vitamins (1/2 MS), and 2% (w/v) sucrose. The medium was solidified using 0.7% plant agar (Duchefa Biochemie). The pH was adjusted to 5.8 before autoclaving. Stock cultures were incubated in constant darkness, in a climatic chamber at 24 ± 1 °C. For induction of SE, only first fronds of 5-month-old plantlets were used as a source of stipe and internode explants (Fig. 1). The initial cultures were maintained on 1/2 MS medium supplemented with 1% (w/v) sucrose, in constant darkness.

Fig. 1 Five-month-old, etiolated sporophyte of *Cyathea delgadii* used as a source of stipe and internode explants for culture initiation; *I* internode explant (the part of sporophyte located below shoot apex and about 1 mm above basal part of the second frond), *S* stipe explant (the part of first frond located 1 mm above shoot apex), *SA* shoot apex, *1st–4th* fronds

Induction of somatic embryogenesis

To determine whether length and diameter of the explant-donor frond affects embryogenic potential, stipe explants (2.5 mm in length) were excised from the first fronds measuring 5, 10, 15, 20 mm in length, and more or less than 300 µm in diameter at their base (marked as < 300 or > 300, respectively). In the case of internode explants, sections measuring 0.5, 1.0, 1.5, 2.0, and 2.5 mm in length were used. Their diameter was similar, regardless of length.

The effect of air desiccation on SE was tested by using a laminar flow cabinet. The stipe and internode explants were exposed to air flow for 15, 30, 45 and 60 min. Also, the effect of sucrose on SE was examined by treating the stipe and internode explants with an aqueous solution of 0.4, 0.5, 0.6 or 0.7 M sucrose for 15, 30, 45 or 60 min. Non-treated explants were used as the control. Sterile water, that was used as an additional control, did not affect SE. Both stress treatments were conducted in the dark, at 24 ± 1 °C. Following stress treatment, explants were maintained on 1/2 MS medium supplemented with 1% (w/v) sucrose, in constant darkness at 24 ± 1 °C.

Evaluation of SE efficiency

The efficiency of SE was calculated as the percentage of responding explants and the number of somatic embryos per responding explant following 1 month of culture for internodes, and 2 months of culture for stipes. The somatic embryo production capacity index (SEPCI) was calculated by multiplying the percentage of responding explants by the number of somatic embryos formed per explant and then dividing the result by 100. In order to evaluate the embryogenic potential of stipe and internode explants, 60 explants for each experiment were examined, and each experiment was repeated three times.

Microscopic examination

The visualisation of SE on the stipe and internode explants was made using an environmental scanning electron microscope (ESEM; FEI QUANTA 200; 0.75 Tr, at a relative humidity of up to 100%, and reduced pressure of less than 10^{-4} Pa).

The explants were also fixed in 2.5% paraformaldehyde (Fluka, Buchs, Switzerland) and 2.5% glutaraldehyde (Sigma, St. Louis, USA) in 0.05 M sodium cacodylate buffer (Fluka) (pH 7.2; room temperature; 24 h) for microscopic analysis. After rinsing in 0.05 M cacodylic buffer, the samples were post-fixed in 2% osmium tetroxide (Carl Roth, Karlsruhe, Germany) in 0.05 M cacodylate buffer at 4 °C for 6 h. Then the explants were dehydrated in a graded ethanol series (30, 50, 70, 90, 96%) for 2 h in each concentration, followed by absolute ethanol and propylene oxide. The samples were infiltrated in a graded Epon epoxy resin (Sigma) mixtures for 48 h in total and transferred into flat embedding molds; the resin polymerised at 65 °C for 16 h. Semi-thin sections (2 µm) were cut using a Leica Ultracut E ultramicrotome (Leica, Wetzlar, Germany) and stained with 0.1% toluidine blue in 1% borax for 15 min. They were examined using a Vanox light microscope (Olympus, Japan) with a computer image analysis system (cellSens Standard ver. 1.7).

Statistical analysis

Statistical analyses were performed using Statgraphics Plus software. Results were expressed as the mean ± SD. The one- or two-way ANOVA analysis of variance and Fisher's least significant difference (LSD) procedure were used. Significance was set at the 0.05 level.

Results

Effect of explant type on embryogenic pathway

Depending upon the explant used for culture initiation, the uni- or multi-cellular type of the embryogenic pathway was observed (Fig. 2). Somatic embryos of single cell origin developed on stipe explants (Fig. 2a–d), whereas on internode explants, the embryos arose from groups of neighbouring cells (Fig. 2e–h).

A short period was required for the expression of SE and somatic embryo formation. The epidermal cells of the stipe began to divide from day 8 of culture (Fig. 2a). Several cell divisions perpendicular to the stipe axis (Fig. 2b) led to the development of numerous somatic embryos at linear stage (Figs. 2c, 3a, b). With increasing frequency of cell divisions occurring within the embryo body, its structure changed from linear to spatial (Fig. 2d). These structures were able to develop a complete functional embryo (Fig. 3c). Neither proliferation of other cells (including the epidermis, cortex and vascular bundle), nor their expansion, or the formation of a meristematic layer or callus, were observed on the surface of stipe explants (Fig. 2c, d) as well as inside it (Fig. 3a, b).

The cells of internodes began to divide at about day 4 of explant culture on the induction medium (Fig. 2e). These divisions increased over time, and after 6 days of culture, many cells of the epidermis were seen to divide on the surface of the explant (Fig. 2f). Together with epidermis, the multiple-divided cortical cells formed a proliferation zone that was composed of several layers of cells with meristematic features, such as small size and isodiametric shape (Fig. 3d, e). Four days later, it was possible to observe somatic embryos of multicellular origin (Figs. 2g, 3f) whose leaf primordia had begun to split at the tip (Fig. 2g,

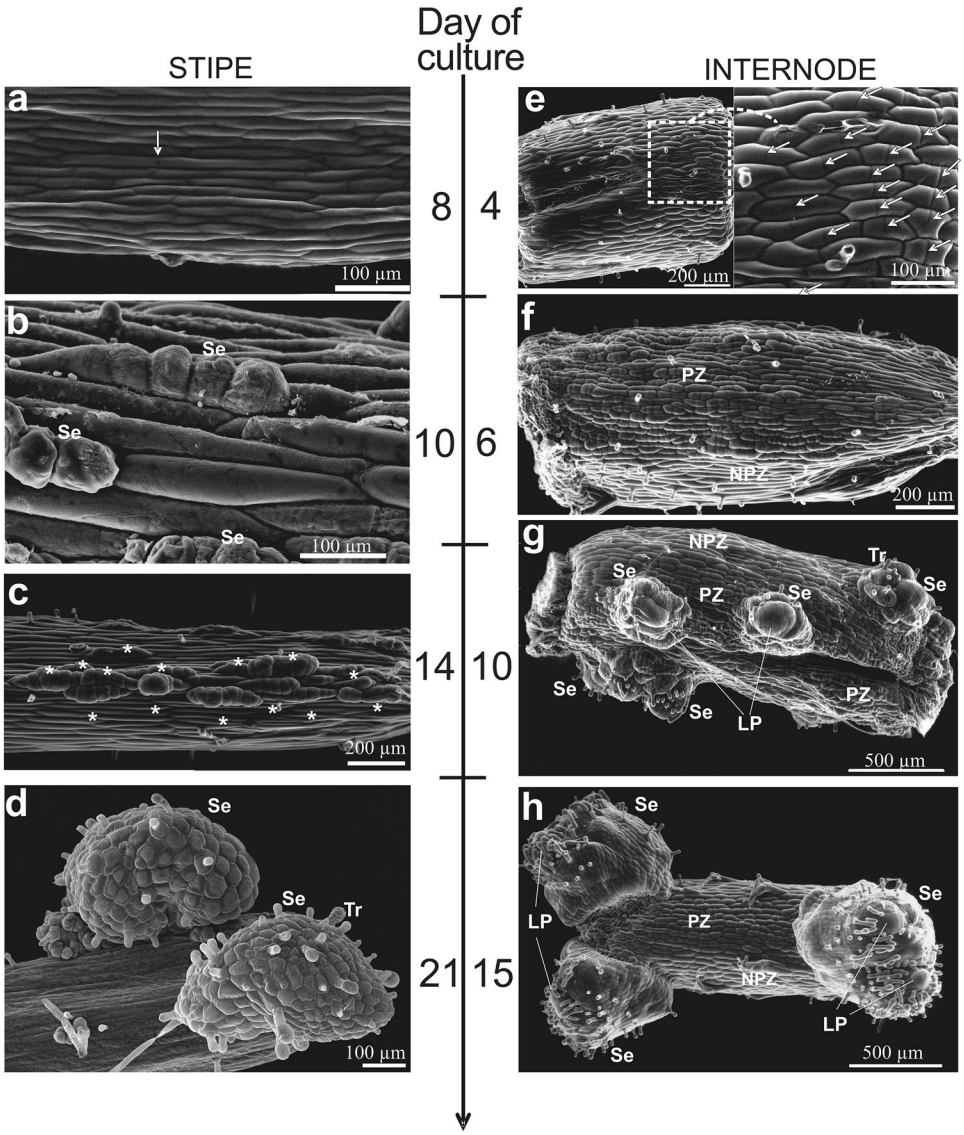

Fig. 2 Pathways of somatic embryo differentiation in stipe (**a–d**) and internode (**e–h**) explants during 21 and 15 days of culture, respectively. Culture conditions: 1/2 MS, 1% sucrose, constant darkness. **a** First cell division of stipe epidermis; **b** pro-embryos following several cell divisions perpendicular to the stipe axis; **c** several somatic embryos at linear developmental stage; **d** somatic embryos of single cell origin at early embryonic leaf stage; **e** first cell divisions of the internode epidermis and enlarged image of proliferation zone (inset). Epidermal cells following one or two cell divisions perpendicular to the axis of the internode. Arrows show cell walls; **f** proliferation zone formed by numerous dividing cells with meristematic features, i.e. small size and isodiametric shape; **g** somatic embryos of multicellular origin bearing leaf primordia; **h** somatic embryos of multicellular origin in contact with the basal area are typically fused to the original explant tissue. Following 15 days of culture, they have fully developed leaf primordia that are protected by numerous trichomes. *Asterisk* somatic embryo, *LP* leaf primordium, *NPZ* non-proliferation zone, *PZ* proliferation zone, *Se* somatic embryo, *Tr* trichome

h). No vascular connection between somatic embryos and explants was observed (Fig. 3d–f). Development of early multicellular embryos into well differentiated somatic embryos was quick and synchronous (Figs. 2h, 3g, h). They had a high capacity for embryonic root formation (Fig. 3h, i). Numerous trichomes were observed on the surface of the embryo body. They were mostly located near leaf primordia (Figs. 2d, h, 3c, g, h).

Somatic embryos originating from internode explants grew much faster than those formed on stipes. In the early stages of development, the somatic embryos were well-defined and presented a typical early embryogenic leaf stage of development in both explant types (Fig. 3c, g, h). Both paths of somatic embryo initiation led to production of complete plantlets without sign of morphological disturbances.

Effect of length and diameter of explant-donor frond on the SE efficiency

The efficiency of SE was dependent on the length and diameter of the first frond used as the source of stipe explants (Table 1). The greatest percentage of explants capable of SE (about 90%) was obtained from fronds measuring < 300 µm in diameter, and 10 or 15 mm in length. The greatest number of somatic embryos was also obtained for these explants (21.4 or 17.9, respectively). Explants collected from fronds measuring > 300 µm in diameter, regardless of length, were characterised by low SE efficiency. The percentage of responding explants was no greater than 34.2%, and the number of somatic embryos per responding explant was no more than 17.1. The value of the SEPCI index was at least threefold greater in fronds

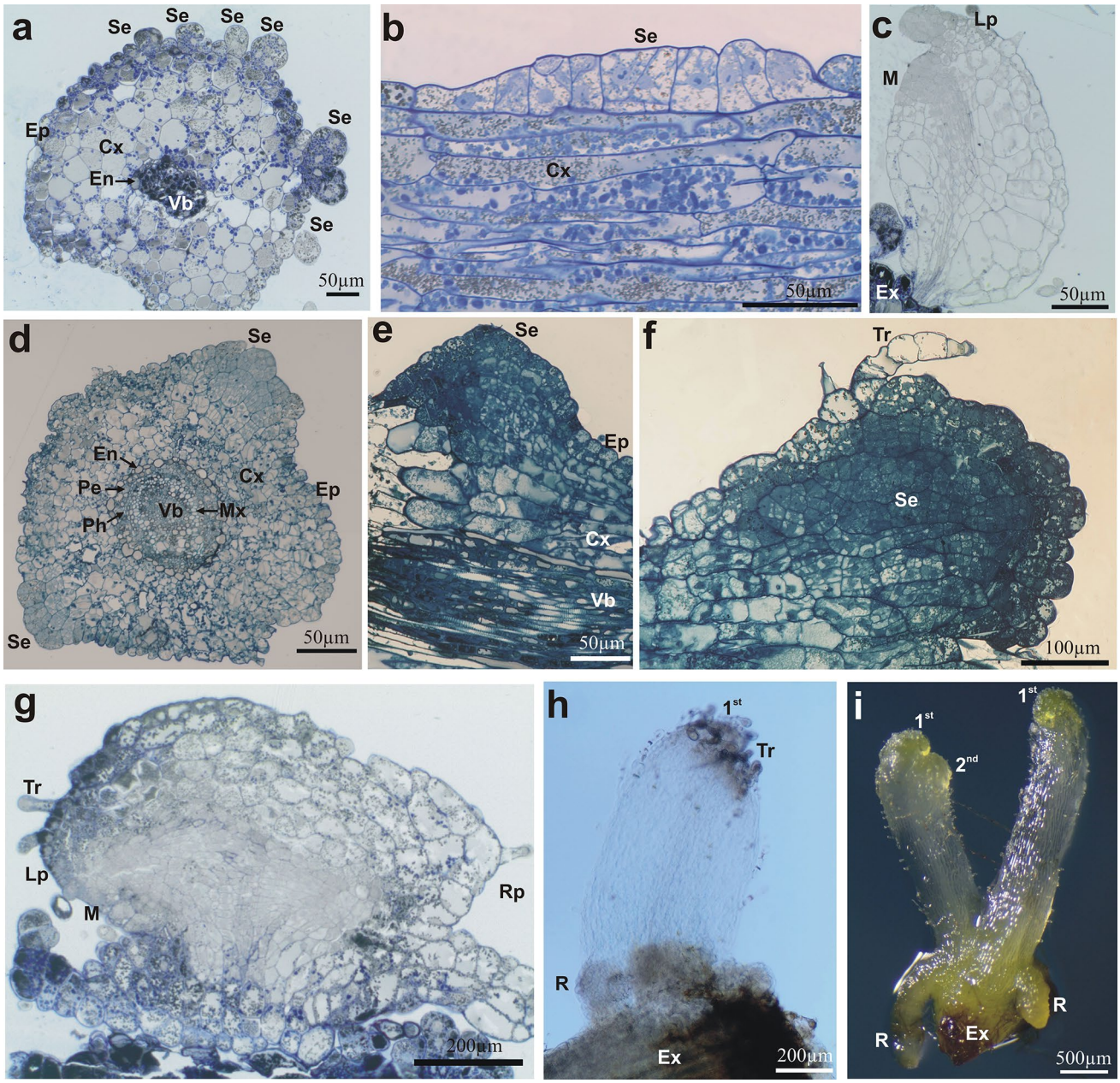

Fig. 3 Morphological evidences for uni- (**a**–**c**) and multicellular (**d**–**i**) origin of somatic embryos in *Cyathea delgadii*. **a** Transverse section of a stipe explant showing divided cells of epidermis that formed somatic embryos, 14th day of culture; **b** longitudinal section of a stipe explant showing linear embryo originating from a single cell of epidermis, 14th day of culture; **c** single cell-origin somatic embryo at early embryonic leaf stage; **d** the multiple-divided epidermal and cortical cells of internode explant (transverse section); 6th day of culture; **e** meristematic primordium emerging on internode explant (longitudinal section); 6th day of culture; **f** embryonic structure formed from cells of the epidermal and cortical layers of internode explant; 10th day of culture (longitudinal section); **g** well differentiated somatic embryo of multicellular origin showing meristematic zone; **h** elongation of embryo body. Presence of trichomes indicates the proximity of developing meristem (cleared in methyl salicilate according to Mikuła et al. 2015b); **i** somatic embryo-derived sporophytes showing the leaf primordia and roots. Semi-thin section of stipe (**a**, **b**) and internode (**d**–**f**) explants, and somatic embryos (**c**, **g**) stained with toluidine blue. Cultures were maintained in constant darkness. *Cx* cortex, *En* endodermis, *Ep* epidermis, *Ex* explant, *Lp* first leaf primordium, *M* shoot apical meristem, *Mx* metaxylem, *Pe* pericycle, *Ph* phloem, *R* root, *Rp* root pole, *Se* somatic embryo, *Tr* trichome, *Vb* vascular bundle, *1st* first leaf, *2nd* second leaf

Table 1 Embryogenic capacity of *C. delgadii* stipe explants taken from the first fronds differing in length and diameter

Basal diameter of the first frond (µm)	Length of the first frond (mm)	% of responding explants	No. somatic embryos/responding explant	SEPCI
> 300	5	25.8 ± 4.7d	13.0 ± 1.1c	3.4
	10	34.2 ± 6.6c	17.1 ± 3.6b	5.8
	15	23.3 ± 5.3d	15.5 ± 2.6bc	3.6
	20	11.7 ± 3.8e	6.9 ± 2.3d	0.8
< 300	5	51.2 ± 11.0b	16.9 ± 2.3b	8.7
	10	91.7 ± 6.2a	21.4 ± 4.8a	19.6
	15	88.7 ± 6.8a	17.9 ± 2.3b	15.9
	20	61.7 ± 4.5b	11.4 ± 4.8c	7.0

SE efficiency was assessed after 2 months of culture in darkness

SEPCI somatic embryo production capacity index, ± SD

Table 2 Effect of internode explant length on the efficiency of SE in *C. delgadii* after 1 month of culture in darkness

Length of the internode explants (mm)	% of responding explants	No. of somatic embryos/responding explants	SEPCI
0.5	95.8 ± 3.7a	2.0 ± 1.0b	2.0
1.0	94.2 ± 6.2a	2.5 ± 0.6a	2.4
1.5	93.3 ± 1.3a	3.1 ± 1.1a	2.9
2.0	91.7 ± 3.1a	2.9 ± 0.3a	2.7
2.5	91.2 ± 1.9a	2.9 ± 0.5a	2.6

SEPCI somatic embryo production capacity index, ± SD

measuring < 300 µm than in those measuring > 300 µm in diameter.

The efficiency of SE did not depend on the length of internode between the first and second frond used as the source of explants (Table 2). The percentage of responding explants in all cases exceeded 91%. A reduced number of somatic embryos (2) and the lowest SEPCI index (2.0) were observed only for the shortest (0.5 mm) internodes. The highest SEPCI index (2.9) was obtained for internode explants measuring 1.5 mm.

Effect of stress treatment on the embryogenic pathway and SE efficiency

To improve the efficiency of SE in *C. delgadii*, the effect of stress treatment was assessed. When stipe explants were subjected to air desiccation for 15–60 min, their capacity to produce somatic embryos became highly suppressed (Table 3). In internode explants, the SE efficiency increased from about three to four somatic embryos following 1 h of air desiccation.

The efficiency of somatic embryo formation was dependent on the explant type, as well as on the concentration and duration of sucrose treatment (Table 4). When internode explants were treated with 0.4 M sucrose for 45 min, more than a fourfold increase in the number of somatic embryos was obtained. The percentage of explants producing somatic embryos compared to that of non-treated internodes was significantly lower. By raising the concentration of sucrose and subjecting explants to a longer period of stress treatment, the SE efficiency became reduced. When stipe explants were used in these experiments, they failed to produce somatic embryos for any of the sucrose concentrations used (Table 4).

The microscopic analysis revealed that somatic embryo formation on the surface of internode explants treated with sucrose solutions occurs in a distinctive manner (Fig. 4).

Table 3 Effect of air desiccation on the efficiency of SE in *C. delgadii*

Time of air desiccation (min)	% of responding explants	No. of somatic embryos/responding explants	SEPCI
Stipe explant			
0	86.7 ± 8.6a	18.2 ± 6.9a	15.8
15	35.0 ± 10.8c	13.5 ± 6.4b	4.7
30	26.7 ± 12.5c	4.8 ± 1.1c	1.3
45	58.3 ± 16.5b	5.5 ± 1.6c	3.2
60	50.0 ± 20.4b	10.0 ± 2.6b	5.0
Internode explant			
0	98.3 ± 2.4b	2.9 ± 0.4c	2.9
15	100 ± 0.0a	3.7 ± 0.6b	3.7
30	100 ± 0.0a	3.6 ± 0.5b	3.6
45	100 ± 0.0a	3.8 ± 0.1b	3.8
60	100 ± 0.0a	4.3 ± 0.4a	4.3

Data were collected after 1 month of culture

Different letters differ significantly at $p \leq 0.05$ according to one-way ANOVA analysis of variance and Fisher's least significant difference (LSD) test

SEPCI somatic embryo production capacity index, ± SD

Table 4 Effect of sucrose treatment on SE efficiency in *C. delgadii*

Sucrose treatment		% of responding explants		No. of somatic embryos/responding explants		SEPCI
Concentration (M)	Duration (min)	I	S	I	S	I
0	0	98.3 ± 2.4a	91.7 ± 6.2a	2.9 ± 0.4e	21.4 ± 4.8a	2.9
0.4	15	95 ± 1.1ab	0b	9.3 ± 0.9b	0b	8.9
	30	95 ± 1.6ab	0b	6.5 ± 0.8d	0b	6.2
	45	90 ± 2.6b	0b	12.1 ± 2.1b	0b	10.9
	60	72.5 ± 2.1c	0b	16.6 ± 1.2a	0b	12
0.5	15	100 ± 2.4a	0b	5.9 ± 1.1d	0b	5.9
	30	70 ± 1.9c	0b	13.7 ± 1.6b	0b	9.6
	45	20 ± 3.1d	0b	19 ± 1.1e	0b	3.8
	60	5 ± 1.1e	0b	12 ± 0.1	0b	0.6
0.6	15	90 ± 3.3b	0b	8.5 ± 1.8c	0b	7.7
	30	20 ± 2.5d	0b	2.9 ± 1.3e	0b	0.6
	45	0f	0b	0f	0b	0
	60	0f	0b	0f	0b	0
0.7	15	90 ± 2.6b	0b	10.3 ± 1.1b	0b	9.25
	30	15 ± 2.2d	0b	16 ± 0.4e	0b	0.4
	45	0f	0b	0f	0b	0
	60	0f	0b	0f	0b	0

Data were collected after 1 month of culture

Different letters differ significantly at $p \leq 0.05$ according to two-way ANOVA analysis of variance and Fisher's least significant difference (LSD) test

SEPCI somatic embryo production capacity index, *I* internode explant, *S* stipe explant, ± SD

As a results of this treatment, somatic embryos of single cell origin developed near structures of multicellular origin (Fig. 4a, b). There was an obvious reduction in the number of somatic embryos of multicellular origin in response to the duration of sucrose treatment. Consequently, only the unicellular pathway of embryo formation was observed when internode explants were treated with sucrose for longer than 30 min (Fig. 4c). Subsequently, both types of structures developed into mature embryos, thereby increasing the yield of this process (Fig. 4d).

Discussion

The pathway and the onset of SE are determined by the physiological and morphological maturity of the plant material source from which the explant derived (Gaj 2004). Initiation of direct SE is restricted to certain responsive cells (Quiroz-Figueroa et al. 2002). According to Williams and Maheswaran (1986), single initials are characteristic of older plant material in which only some epidermal cells are still immature. These cells remain in the predetermined embryogenic condition. The multiple-cell initiation of SE is thought to be a consequence of the explant containing several neighbouring cells in the embryogenic state (Puigderrajols et al. 2001). In our studies, we provide for the first time the structural evidence for the multicellular origin of somatic embryos in fern. We demonstrated that the somatic embryos of *C. delgadii* may develop not only from single epidermal cells, but also from clusters of rapidly dividing cells of epidermis and cortex. The pathway of embryo differentiation was closely related to the explant type used for culture initiation. Somatic embryos derived from stipes of in vitro-grown sporophytes followed a unicellular pathway of direct SE, whereas those derived from internodes followed a multicellular pathway. We also showed that both the activation of cell divisions and the formation of somatic embryos occurred earlier in the culture of internodes than in that of stipe explants. Such behaviour may be a consequence of the different degree of cell maturity of these explants and different content of endogenous hormones (Michalczuk et al. 1992; Centeno et al. 1996; Grzyb et al. 2017). In *C. delgadii*, high frequency of cell divisions in the epidermal and cortical layers of internode explants, and the capability of many neighbouring cells to act in a coordinated manner led to the differentiation and rapid embryo development. We speculate that this may be the main reason why somatic embryo formation via the multicellular pathway occurs very quickly.

Although both investigated explant types of *C. delgadii* were able to produce somatic embryos, the morphogenic capacity of internodes was much lower than stipes. Almost 92% of stipe explants excised from the first frond, measuring

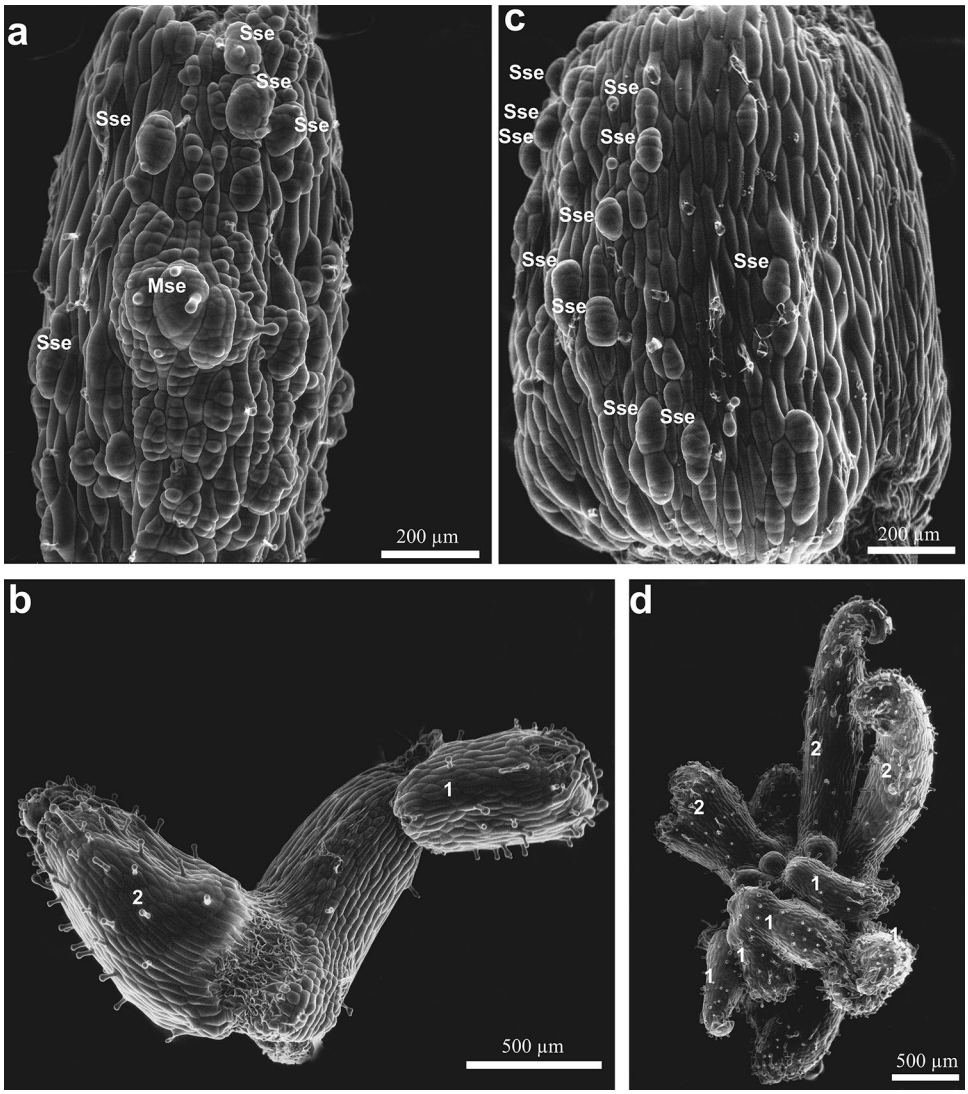

Fig. 4 Modification by sucrose treatment of pathways of somatic embryogenesis on internode explants of *Cyathea delgadii* (scanning electron micrographs). **a** Somatic embryos that have developed along unicellular and multicellular pathways following treatment with 0.4 M sucrose for 30 min after 12 days of culture and **b** 20 days of culture. **c** Numerous somatic embryos that have developed along the unicellular pathway following the treatment of internode explants with 0.5 M sucrose for 45 min (day 12 of culture). **d** Numerous somatic embryo-derived sporophytes obtained from internode explants treated with 0.4 M sucrose for 30 min after 30 days of culture. *Mse* multicellular origin of somatic embryo, *Sse* single cell origin of somatic embryo, *1* juvenile sporophyte derived from a single epidermal cell; *2* juvenile sporophyte derived from several explant cells

10 mm in length and less than 300 µm in diameter, produced, on average, 21 somatic embryos. As a result of frequent cell divisions, the groups of embryogenic-competent cells of internode explants formed protuberances that gave rise to a maximum of three somatic embryos. Similarly, a lower percentage of explants producing somatic embryos was observed for multicellular SE than for unicellular SE in *Theobroma cacao* L. (Maximova et al. 2002). However, in this species, the difference in SE efficiency was due to severe embryonic malformations that led to the formation of abnormal somatic embryos or embryo-like structures. In the absence of abnormalities during the development of somatic embryos in *C. delgadii*, it would appear that the competition may have prevented further development of some embryogenic cells in favour of others, as was proposed by Jones and Rost (1989).

In *C. delgadii*, beside the environmental factors (such as the mineral and organic nutrients of culture medium, or light), some explant features can affect the efficiency of SE (Mikuła et al. 2015a). The key factor to induction of the embryogenic capacity is the etiolation of fern sporophytes that are the source of plant material (Mikuła et al. 2015a). Our previous studies also showed that SE induced on stipes can be effectively stimulated by the length of explants (Mikuła et al. 2015a), however, the factor is ineffective in the case of internode explants. In this study, the donor fronds differing in their length and diameter were found to have a profound influence on SE of *C. delgadii*. Short and thin fronds were found to be more effective in the production of somatic embryos, and it may be related to their age. The positive effect of immature tissues or relatively young individuals on SE as described here is consistent with the earlier observations made with *Trifolium repens* (Maheswaran and Williams 1985) or *Eucalyptus camaldulensis* (Prakash and Gurumurthi 2010).

Induction of SE on the internode explants of *C. delgadii* can be a good example of stress-related plant response. We revealed that stress treatment can alter the pathway of somatic embryo differentiation and affect embryogenic capacity. The SE efficiency of internode explants was significantly improved by short-term treatment (up to 1 h) with sucrose. There is evidence in the literature to support the hypothesis that osmotic treatment may enhance somatic embryo production in two ways: it may cause selection of certain cell types, as shown for osmotically dehydrated embryogenic tissue of *Gentiana cruciata, G. kurroo* (Mikuła et al. 2011a, b) and *Pinus sylvestris* (Häggman et al. 1998), or alternatively, severe but non-fatal stress treatment may lead to the symplasmic isolation of cells (Marzec and Kurczynska 2014). It was demonstrated that disruption of plasmodesmata between explant cells promotes the somatic-to-embryogenic transition (Verdeil et al. 2001; You et al. 2006). In *C. delgadii*, changing the pathway of the origin of somatic embryos from multicellular to unicellular supports the view that the acquisition of embryogenic competence is preceded by cell isolation in sucrose-treated internode explants. This treatment has enabled the establishment of a new tissue culture method for inducing efficient SE in *C. delgadii*.

In summary, the results presented in this paper together with earlier studies (Mikuła et al. 2015a) help to broaden our knowledge of factors influencing efficiency of SE in monilophytes. They also allow us to trace the pathway of somatic embryo origin that is determined by the explant type and short-term sucrose treatment. This stress treatment improves the efficiency of direct SE by replacing the multicellular origin of somatic embryos with unicellular pathway. These findings open new possibilities for studying mechanisms of expression of embryogenic totipotency associated with a single- and multiple-cell origin of somatic embryos, especially since both pathways occur via direct SE without involving intermediate callus, on hormone-free medium.

Acknowledgements The authors thank Dr. Mirosław Sobczak (Warsaw University of Life Sciences—SGGW, Poland) for his help during microscopic analysis, and Professor Jan J. Rybczyński (PAS Botanical Garden - CBDC in Powsin, Warsaw, Poland) for valuable advices and creative discussion during preparation of the manuscript. This research was supported by the Polish National Science Centre (NCN), No. 2017/27/N/NZ3/00434.

Author contributions MG and AM conceived and designed the experiments, wrote the paper. MG performed the experiments and analysed the data.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Warszawa, 26.05.2020

Polska Akademia Nauk Ogród Botaniczny – Centrum
Zachowania Różnorodności Biologicznej w Powsinie

Oświadczenie doktorantki

W związku z wykorzystaniem poniżej wymienionych publikacji jako elementu rozprawy doktorskiej, oświadczam, iż mój udział w ich powstaniu polegał na:

Publikacja nr 1

Grzyb M, Kalandyk A, Waligórski P, Mikuła A 2017 The content of endogenous hormones and sugars in the process of early somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb. Plant Cell, Tissue and Organ Culture 129:387-397

- przygotowaniu materiału roślinnego do analizy zawartości hormonów i cukrów; udziale w uzyskaniu ekstraktów do ich oznaczania; wizualizacji i opracowaniu wyników oraz przygotowaniu manuskryptu. Jako autor korespondencyjny prowadziłam korespondencję z redakcją czasopisma.

Publikacja nr 2

Grzyb M, Kalandyk A, Mikuła A (2018) Effect of TIBA, fluridone and salicylic acid on somatic embryogenesis and endogenous hormone and sugar contents in *Cyathea delgadii* Sternb. Acta Physiologiae Plantarum 40:1

- współudziale w opracowaniu koncepcji analiz; przygotowaniu materiału roślinnego do analizy zawartości hormonów i cukrów; przeprowadzeniu eksperymentów z wykorzystaniem inhibitorów; analizie, wizualizacji i opracowaniu wyników oraz przygotowaniu manuskryptu. Jako autor korespondencyjny prowadziłam korespondencję z redakcją czasopisma.

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Publikacja nr 3

Grzyb M, Wróbel-Marek J, Kurczyńska E, Sobczak M, Mikuła A 2020 Symplasmic isolation contributes to somatic embryo induction and development in the tree fern *Cyathea delgadii* Sternb. Plant and Cell Physiology. doi: 10.1093/pcp/pcaa058

- współudziale w opracowaniu koncepcji analiz; przygotowaniu materiału roślinnego do wszystkich eksperymentów; wykonaniu analizy ESEM; wykonaniu skrawków semicienkich i przeprowadzeniu ich analizy w mikroskopie świetlnym; przygotowaniu preparatów i udziale w prowadzeniu analizy symplastowej komunikacji; wizualizacji i opracowaniu wyników oraz przygotowaniu manuskryptu. Jako autor korespondencyjny prowadziłam korespondencję z redakcją czasopisma.

Publikacja nr 4

Grzyb M, Mikuła A (2019) Explant type and stress treatment determine the uni- and multicellular origin of somatic embryos in the tree fern *Cyathea delgadii* Sternb. Plant Cell, Tissue and Organ Culture 136:221–230

- współudziale w opracowaniu koncepcji analiz; przygotowaniu materiału roślinnego do analiz mikroskopowych; przeprowadzeniu obserwacji ESEM oraz preparatów semicienkich w mikroskopie świetlnym; przeprowadzeniu eksperymentów z wykorzystaniem roztworów sacharozy i desykacji powietrznej; wizualizacji i opracowaniu wyników, przygotowaniu manuskryptu. Jako autor korespondencyjny prowadziłam również korespondencję z redakcją czasopisma.

Małgorzata Grzyb

Mgr Małgorzata Grzyb

Prof. dr hab. Anna Mikuła

Warszawa, 25.05.2020

Polska Akademia Nauk Ogród Botaniczny – Centrum
Zachowania Różnorodności Biologicznej w Powsinie

Oświadczenie

W związku z wykorzystaniem przez mgr Małgorzatę Grzyb poniżej wymienionych publikacji jako elementu rozprawy doktorskiej, oświadczam, iż mój udział w ich powstaniu, jako promotora polegał na opracowaniu koncepcji badań oraz współpracy w zakresie analizy wyników i przygotowania manuskryptu.

Publikacja nr 1

Grzyb M, Kalandyk A, Waligórski P, Mikuła A (2017) The content of endogenous hormones and sugars in the process of early somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb. Plant Cell Tissue and Organ Culture 129:387-397

Publikacja nr 2

Grzyb M, Kalandyk A, Mikuła A (2018) Effect of TIBA, fluridone and salicylic acid on somatic embryogenesis and endogenous hormone and sugar contents in *Cyathea delgadii* Sternb. Acta Physiologiae Plantarum 40:1

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Grzyb M, Wróbel-Marek J, Kurczyńska E, Sobczak M, Mikuła A (2020) Symplasmic isolation contributes to somatic embryo induction and development in the tree fern *Cyathea delgadii* Sternb. Plant and Cell Physiology. doi: 10.1093/pcp/pcaa058

Publikacja nr 4

Grzyb M, Mikuła A (2019) Explant type and stress treatment determine the uni- and multicellular origin of somatic embryos in the tree fern *Cyathea delgadii* Sternb. Plant Cell, Tissue and Organ Culture 136:221-230

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Kraków, 22.05.2020

Oświadczenie

W związku z wykorzystaniem przez mgr Małgorzatę Grzyb poniżej wymienionych publikacji jako elementu rozprawy doktorskiej, oświadczam, iż mój udział w ich powstaniu polegał na:

1) przeprowadzeniu rozdziału i oznaczenia zawartości endogennych cukrów z wykorzystaniem HPLC

Publikacja nr 1

Grzyb M, Kalandyk A, Waligórski P, Mikuła A (2017) The content of endogenous hormones and sugars in the process of early somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb. Plant Cell Tissue and Organ Culture 129:387-397

2) przeprowadzeniu rozdziału i oznaczenia zawartości endogennych hormonów i cukrów z wykorzystaniem HPLC

Publikacja nr 2

Grzyb M, Kalandyk A, Mikuła A (2018) Effect of TIBA, fluridone and salicylic acid on somatic embryogenesis and endogenous hormone and sugar contents in Cyathea delgadii Sternb. Acta Physiologiae Plantarum 40:1

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Kraków, 21.05.2020

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Oświadczenie

W związku z wykorzystaniem przez mgr Małgorzatę Grzyb poniżej wymienionej publikacji jako elementu rozprawy doktorskiej, oświadczam, iż mój udział w jej powstaniu polegał na przeprowadzeniu rozdziału i oznaczenia zawartości endogennych hormonów z wykorzystaniem HPLC oraz opisu metodyki z tego zakresu badań.

Publikacja nr 1

Grzyb M, Kalandyk A, Waligórski P, Mikuła A (2017) The content of endogenous hormones and sugars in the tree fern *Cyathea delgadii* Sternb. Plant Cell Tissue and Organ Culture 129:387-397

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Oświadczenie

W związku z wykorzystaniem przez mgr Małgorzatę Grzyb poniżej wymienionej publikacji jako elementu rozprawy doktorskiej, oświadczam, że mój udział w jej powstaniu dotyczył opracowania koncepcji badań, umożliwienia wykorzystania mikroskopu konfokalnego (sprzętu należącego do byłego Wydziału Biologii i Ochrony Środowiska) do analiz komunikacji symplastowej, współpracy w zakresie analizy wyników oraz przygotowania manuskryptu.

Publikacja nr 3

Grzyb M, Wróbel-Marek J, Kurczyńska E, Sobczak M, Mikuła A (2020) Symplasmic isolation contributes to somatic embryo induction and development in the tree fern *Cyathea delgadii* Sternb. Plant and Cell Physiology. doi: 10.1093/pcp/pcaa058

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Oświadczenie

W związku z wykorzystaniem przez mgr Małgorzatę Grzyb poniżej wymienionej publikacji jako elementu rozprawy doktorskiej, oświadczam, iż mój udział w jej powstaniu polegał na przeprowadzeniu wizualizacji przemieszczania fluorochromów transportu symplastowego z wykorzystaniem mikroskopu konfokalnego, współpracy w zakresie analizy i wizualizacji wyników oraz przygotowania manuskryptu.

Publikacja nr 3

Grzyb M, Wróbel-Marek J, Kurczyńska E, Sobczak M, Mikuła A (2020) Symplasmic isolation contributes to somatic embryo induction and development in the tree fern *Cyathea delgadii* Sternb. Plant and Cell Physiology, doi:10.1093/pcp/pcaa058

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Oświadczenie

W związku z wykorzystaniem przez mgr Małgorzatę Grzyb poniżej wymienionej publikacji jako elementu rozprawy doktorskiej, oświadczam, iż mój udział w jej powstaniu polegał na przeprowadzeniu analizy materiału roślinnego z wykorzystaniem transmisyjnego mikroskopu elektronowego oraz współpracy w przygotowaniu końcowej wersji manuskryptu.

Publikacja nr 3

Grzyb M, Wróbel-Marek J, Kurczyńska E, Sobczak M, Mikuła A (2020) Symplasmic isolation contributes to somatic embryo induction and development in the tree fern *Cyathea delgadii* Sternb. Plant and Cell Physiology. doi: 10.1093/pcp/pcaa058

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